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Production, Stabilization, and Immobilization of δ-(L-α-Aminoadipyl)-L-Cysteinyl-D-Valine (ACV) Synthetase from *Streptomyces clavuligerus*; Refolding and Characterization of the Domain-1 Polypeptide

by

Tenshuk Ange Kadima

©

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SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA
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Date: 16th September 1993

DEDICATION.

I dedicate this thesis
to my wife, Mavis
and
to our children, Elenga, Daniel, and Shalla
for enriching my life with Joy and Love
and
for giving a meaning to my endeavors to obtain this degree.

ABSTRACT.

δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine (ACV)-synthetase from *Streptomyces clavuligerus* was studied as a possible alternative to chemical synthesis of ACV. Analyses of the culture harvest time and stabilization conditions were carried out to optimize the enzyme yield. ACV-synthetase was produced early in the growth phase and its activity remained high up to 96 h of growth. A progressive decrease in the stability of crude ACV-synthetase was observed with increasing culture age during storage at 4°C. Using ACV-synthetase isolated from 30-40 h cultures, the presence of dithiothreitol, the three substrate amino acids and glycerol increased the enzyme stability. This stabilized enzyme retained half of its initial activity after 6 days at 4°C.

Studies on the mechanism of the reaction catalyzed by ACV-synthetase using ^{32}P-ATP indicated that the ATP-dependent synthesis of ACV proceeds with a concomitant formation of AMP. Reactions carried out with individual amino acids revealed that AMP formation is highest with cysteine and hardly detectable with L-α-aminoadipate, consistent with previous studies on the ATP/^{32}P-pyrophosphate exchange activity of this enzyme. The Michaelis constants of the purified enzyme were 0.43, 3.75, 6.25, and 7.58×10^{-4} M for L-cysteine, L-valine, L-α-aminoadipate and ATP, respectively.

Studies on continuous ACV synthesis were done using the enzyme immobilized by physical confinement in a selectively-permeable reactor. This process gave higher specific activity than binding to DEAE-Trisacryl, and no activity was observed with enzyme covalently bound to insoluble supports. By maintaining a nitrogen atmosphere in the reactor, both enzyme and product stabilities were increased. After several repeated uses, the enzyme retained 45 to 65% of its initial activity after 24 h. Using partially purified enzyme, ACV production was stimulated more by combinations of phosphoenolpyruvate and ATP than by higher concentrations of ATP alone: this effect was not observed with purified enzyme. ACV synthetase was shown to convert up to 30% of the reaction components based on the limiting cysteine concentration. Reaction products were investigated as potential inhibitors. Of these, AMP was the most inhibitory, but only at concentrations in excess of those formed in reaction mixtures on the basis of the amount of ACV produced. Based on the highest substrate conversion achieved in a 24-h operation of a small reactor and the stability of the enzyme, the same batch of enzyme in a 1-L reactor was capable of producing 460 mg ACV.

Investigations on the production and characterization of the putative domain-1 of ACV-synthetase were carried out with a previously cloned 3-kb DNA fragment of ACV-synthetase gene. The domain-1 polypeptide was overexpressed in *Escherichia coli* JM109 as insoluble and inactive inclusion bodies, constituting 21% of total cellular protein. Optimized inclusion body solubilization conditions (9 M urea and 5% or less glycerol in a the denaturation buffer), and protein refolding conditions (quick dilution of the unfolded protein in Tris buffer pH 8.5, and overnight incubation at 10°C) were found to reactivate ACV-synthetase domain-1. Treatment of the refolded protein with 0.05% sodium dodecyl sulfate prevented individual polypeptide units from reassociation. ACV-synthetase domain-1 was found to be functionally active in binding L-α-aminoadipate but not L-cysteine or L-valine, representing the first evidence at the protein level for the existence of separate domains for the activation of individual amino acids in ACV-synthetase.

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I would like to offer my heartfelt gratitude to my supervisors Drs. M.A. Pickard and S.E.Jensen for all their help and encouragement during the course of this research project. Their keen insights and timely advice were crucial to the successful completion of my thesis. I would also like to thank all the members of my committee, whose constructive criticism on my work, and helpful advice on academic matters, were of great importance for the completion of this thesis.

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LIST OF ABBREVIATIONS.

Abbreviation	Description
AAA	α-aminoadipic acid
ACV	δ-(L-α-aminoadipyl)- L-cysteinyl-D-valine
ACVSR	ACV-synthetase related protein
ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
APA	aminopenicillanic acid
ATP	adenosine-5'-triphosphate
CM	carboxymethyl
CPG	controlled-pore glass
CoA	coenzyme A
DAC	deacetylcephalosporin C
DAOC	deacetoxycephalosporin C
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FPLC	Fast Protein Liquid Chromatography
HPLC	High Performance Liquid Chromatography
IPNS	isopenicillin N synthase
kDa	kilodalton
LSC	Liquid Scintillation Counter
MDKG	MOPS plus DTT plus KCl plus glycerol
MOPS	3-(N-Morpholino)propane-sulfonic acid
MW	molecular weight
PEI	polyethyleneimine
PEP	phosphoenolpyruvate
P_i	inorganic phosphate
PP_i	inorganic pyrophosphate
rpm	revolution/min
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
TCS/S	Trypticase Soy broth plus 1% (w/v) starch
TDEG	Tris buffer plus DTT, plus EDTA plus glycerol
Tris	Tris(hydroxymethyl)aminomethane

1. INTRODUCTION.

1.1 Biosynthesis and regulation of ß-lactam antibiotics.

The initial discovery of the ß-lactam penicillin from Fleming's contaminating *Penicillium* fungus and its remarkable antibacterial properties now date back more than 60 years (1929). Yet research continues to be devoted to the isolation and development of new ß-lactam antibiotics (Kleinkauf and von Döhren, 1992; Ono and Harada, 1990; Page, 1992), a direct reflection of their clinical effectiveness against bacteria combined with low toxicities towards mammals. At present, about 150 microbial antibiotic compounds with economic significance are produced on a large scale by fermentation. This is a small proportion of the more than 7000 natural microbial antibiotics, many of which have been discovered by ingenious screening methods (Demain, 1983; Vandamme, 1984, 1990). Of these industrial antibiotics, ß-lactam antibiotics account for greater than 50% of the worldwide consumption and seem likely to remain an important factor in the control of infectious diseases (Jensen, 1986).

In recent years, intensive efforts have been focussed on identifying and characterizing the regulatory mechanisms and genetic systems that determine penicillin and cephalosporin biosynthesis. Significant progress has been made from the application of recombinant DNA technology in this field, which has turned the molecular genetics of ß-lactam biosynthesis into one of the most challenging and rapidly expanding areas of research in bioactive microbial products. To a large extent the chemical nature of these clinically important compounds, their mode of action, and the biochemical pathways by which they are assembled are now well understood (Aharonowitz *et al.*, 1992; Page, 1992).

Several articles and books document these studies in considerable detail (Aharonowitz *et al.*, 1992; Baldwin and Abraham, 1988; Demain, 1992; Demain and Solomon, 1983; Ingolia and Queener, 1989; Jensen, 1986; Kleinkauf and von Döhren, 1990a; Miller and Ingolia, 1989; Page, 1992; Queener, 1990; Turner, 1992). This review will focus on certain aspects of (i) the biosynthetic pathways and regulation of cephamycin and cephalosporin production; (ii) the physiological and biochemical studies of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV)-synthetase; (iii) the production of antibiotics and precursor molecules by immobilized enzymes; and finally (iv) the characteristics of the recombinant DNA technology applicable to studies of antibiotic biosynthetic enzymes. This chapter will also include a summary of the objectives of this research project.

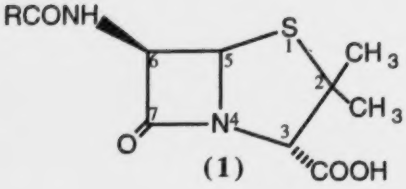
1.1.1 ß-Lactam antibiotics and producing organisms.

ß-Lactam antibiotics were originally discovered in filamentous fungi, then subsequently found to be produced by a much larger group of microorganisms (Elander, 1983). Chief among these are mycelium forming Gram-positive bacteria belonging to the genus *Streptomyces* and some unicellular Gram-negative bacteria. Recently, it has been claimed that ß-lactams are also produced intracellularly by members of the genera *Bacillus, Enterobacter, Staphylococcus, Streptococcus* and even *Escherichia coli* (Allison, 1990). This family of antibiotics is represented by a diverse array of chemical structural types (Page, 1992), characterized by the possession of the four-membered ß-lactam ring (Fig. 1.1). They are classified into several groups according to their various structures (Cooper, 1992; Jungheim and Ternansky, 1992; Page, 1992), and despite this chemical diversity, the ß-lactam moiety present in each is capable of acylating the penicillin binding protein thus interfering with the bacterial cell-wall biosynthesis (Jungheim and Ternansky, 1992). The classical ß-lactam structures include the penicillins (**1**), cephalosporins (**2**) and cephamycins (**3**) (Colvin, 1992), which contain the ß-lactam ring fused to a second sulfur-containing ring: thiazolidine in penicillins, and dihydrothiazine in cephalosporins and cephamycins.

1.1.2 Biosynthetic pathways of the classical ß-lactam antibiotics.

Until relatively recently, progress in the understanding of the machinery responsible for the biosynthesis of classical ß-lactams has been slow. In the last decade

Figure 1.1 Structures of different classes of naturally occurring ß-lactam antibiotics. Penams (**1**), cephems [cephalosporins (**2**) and cephamycins (**3**)], oxacephems (**4**), penems (**5**), oxapenams (**6**), carbapenems (**7**), nocardicins (**8**), and monobactams (**9**).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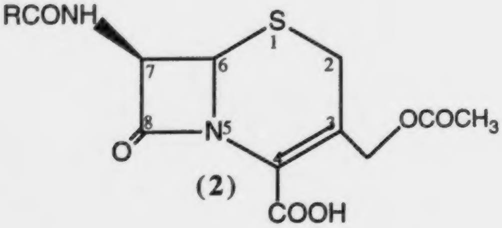
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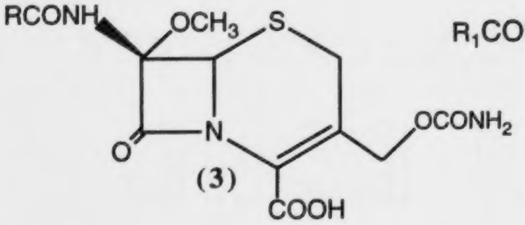
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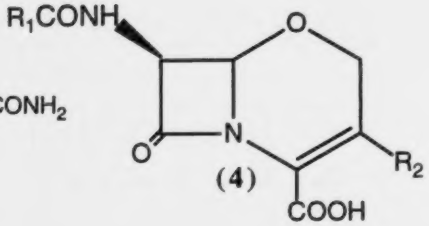
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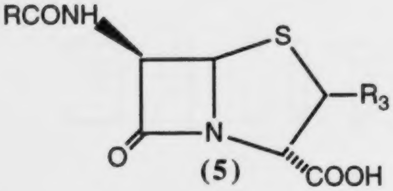
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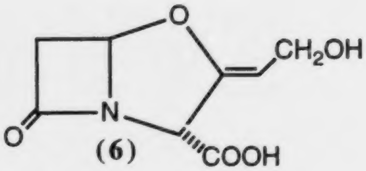
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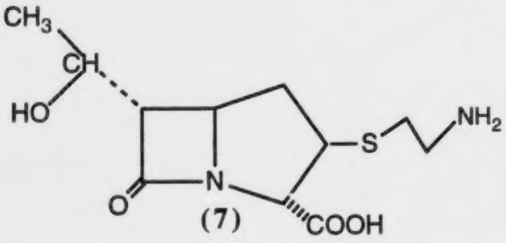
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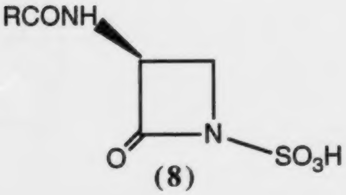
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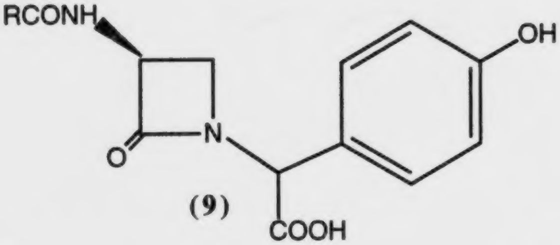
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(9)

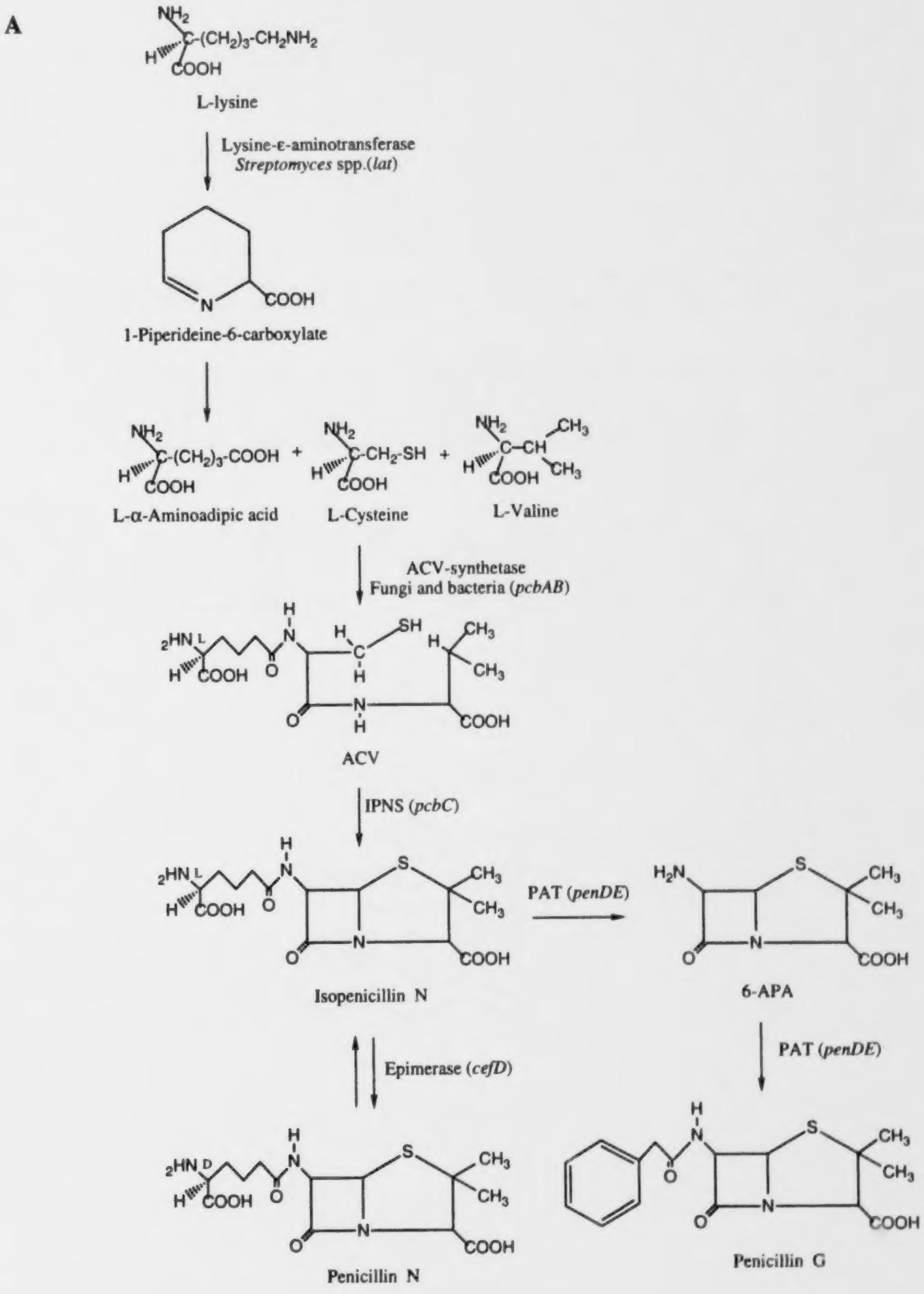
however, many interesting developments have taken place within this area of research, making it undoubtedly the most widely studied and best understood secondary metabolic process to date (Vining *et al.,* 1990). The current knowledge of the biosynthetic pathways leading to the penicillins, cephalosporins and cephamycins has been recently reviewed (Baldwin and Schofield, 1992; Demain and Piret, 1991; Jensen and Demain, 1993; Martín and Liras, 1989) and is summarized in Figure 1.2. This is a composite picture constructed with data obtained from many microorganisms, and no single microorganism can carry out the entire series of reactions (Demain, 1983). According to the figure, antibiotics with the penam and cephem structures all appear to be formed by the same biosynthetic pathway which, at an early stage, assembles the three amino acids L-α-aminoadipic acid, L-cysteine, and L-valine into the tripeptide ACV, and subsequently converts ACV into isopenicillin N. The production of isopenicillin N represents a branching point between those organisms that produce penicillins only or cephalosporins, at a later stage in the biosynthetic pathways (Baldwin and Schofield, 1992). The cephalosporin pathways go through deacetylcephalosporin, a point at which the subsequent reactions start to vary in the different producing organisms. A brief account of these reaction steps, relevant genetic studies and biochemical properties of the enzymes involved is presented in the following sections, except for studies on ACV-synthetase which will be reviewed in detail in section 1.2 below, because this enzyme is the focal point in the work reported in this thesis.

1.1.2.1 Synthesis of L-α-aminoadipic acid.

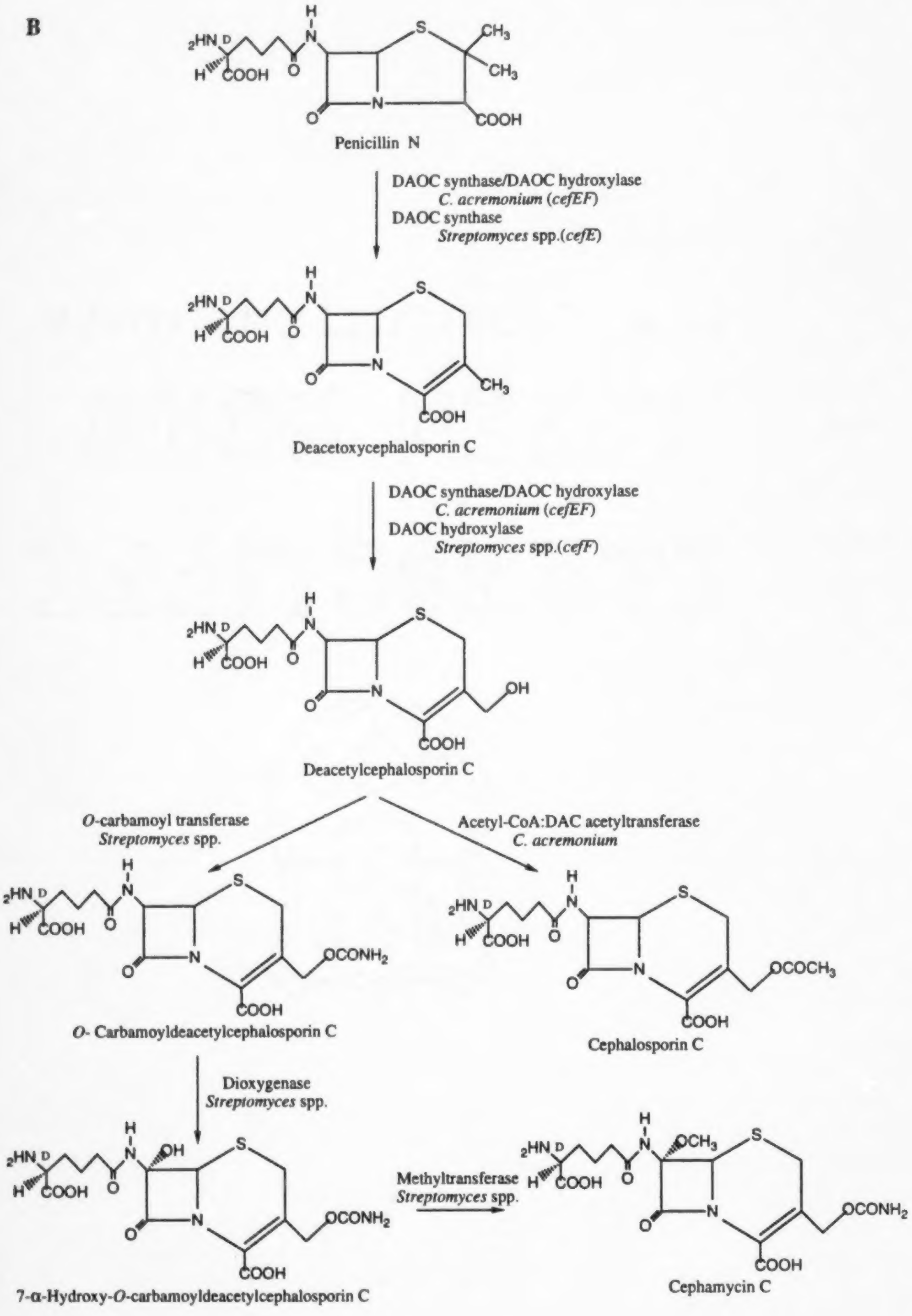
The side chain of many naturally occurring classical ß-lactams is derived from L-α-aminoadipic acid (L-AAA). The formation of this amino acid in prokaryotic organisms differs strikingly from that in filamentous fungi (Martín and Aharonowitz, 1983; Vining *et al.*, 1990). In eukaryotes such as *Cephalosporium acremonium, Aspergillus nidulans*, and *Penicillium chrysogenum*, L-AAA is an obligate intermediate in the homocitrate pathway of lysine synthesis. However, in prokaryotes such as *Streptomyces lipmanii* and *Nocardia*

Figure 1.2 Biosynthetic pathways leading to (**A**) the penicillins and (**B**) the cephalosporins and cephamycins.

ACV = δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine, IPNS = isopenicillin N synthase, DAOC = deacetoxycephalosporin C , DAC = deacetylcephalosporin C, 6-APA = 6-aminopenicillanic acid, PAT = isopenicillin N/phenylacetyl-CoA:6-APA acyltransferase. Also shown are the names in parentheses of the various genes encoding the biosynthetic enzymes that have been identified.


A
L-lysine
Lysine-ε-aminotransferase
Streptomyces spp.(lat)
1-Piperideine-6-carboxylate
L-α-Aminoadipic acid
L-Cysteine
L-Valine
ACV-synthetase
Fungi and bacteria (pcbAB)
ACV
IPNS (pcbC)
Isopenicillin N
PAT (penDE)
6-APA
PAT (penDE)
Epimerase (cefD)
Penicillin N
Penicillin G

B


Penicillin N
DAOC synthase/DAOC hydroxylase
C. acremonium (*cefEF*)
DAOC synthase
Streptomyces spp.(*cefE*)
Deacetoxycephalosporin C
DAOC synthase/DAOC hydroxylase
C. acremonium (*cefEF*)
DAOC hydroxylase
Streptomyces spp.(*cefF*)
Deacetylcephalosporin C
O-carbamoyl transferase
Streptomyces spp.
Acetyl-CoA:DAC acetyltransferase
C. acremonium
O- Carbamoyldeacetylcephalosporin C
Cephalosporin C
Dioxygenase
Streptomyces spp.
Methyltransferase
Streptomyces spp.
7-α-Hydroxy-*O*-carbamoyldeacetylcephalosporin C
Cephamycin C
$_2$HN
D
H
COOH
O
N
S
CH$_3$
OH
OCONH$_2$
OCOCH$_3$
OCH$_3$

(previously *Streptomyces*) *lactamdurans*, L-AAA is derived from the breakdown of lysine via a pathway involving lysine ε-aminotransferase, which exclusively supplies L-AAA for ß-lactam synthesis (Kern *et al.*, 1980; Madduri *et al.*, 1989). This route is now being considered as a secondary metabolic pathway in actinomycetes (Tobin *et al.*, 1991), and several pieces of evidence from recent studies are in agreement with this view (Kern *et al.*, 1980; Kovacevic *et al.*, 1989; Madduri *et al.*, 1989, 1991a,b; Miller and Ingolia, 1989; Smith *et al.*, 1990).

Characterization of the L-AAA pathway involving lysine ε-aminotransferase in *N. lactamdurans* suggested that the conversion of L-AAA from lysine involves two reaction steps. First, lysine ε-aminotransferase catalyzes the conversion of lysine to 1-piperideine-6-carboxylate via ε-deamination of L-lysine; and then 1-piperideine-6-carboxylate is presumably oxidized to form L-AAA in a nicotinamide adenine dinucleotide-linked enzymic oxidation reaction (Kern *et al.*, 1980). Lysine ε-aminotransferase from *N. lactamdurans* has an activity pH optimum of 7.1, a broad temperature optimum of 25 to 35°C, a specific requirement for α-ketoglutarate as the amino group receptor, and a Michaelis constant for lysine of 3×10^{-3} M (Kern *et al.*, 1980). The *S. clavuligerus* lysine ε-aminotransferase is a single polypeptide with a molecular weight (MW) of about 49 kDa (Tobin *et al.*, 1991).

1.1.2.2 Formation of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine.

The channelling of primary metabolites towards classical ß-lactams in both eukaryotes and prokaryotes begins with the formation of ACV, a tripeptide initially discovered by Arnstein and Morris (1960) in *P. chrysogenum*. In eukaryotes, this reaction step constitutes a genuine point of entry of the primary metabolites, L-AAA, L-cysteine, and L-valine in the penicillin and cephalosporin biosynthetic pathways; whereas in prokaryotes, L-AAA may not be considered as a primary metabolite as mentioned above. However, in a broader sense, ACV can be regarded as the key intermediate, because of the central role this compound has been shown to play in the biosynthesis of all penicillins and cephalosporins. This tripeptide has been isolated and identified unequivocally from a number of microorganisms including *C. acremonium*, *S. clavuligerus*, *N. lactamdurans*, and *Paecilomyces persicinus* (Martín and Liras, 1989). Studies on the formation of ACV have also shown a good correlation between the tripeptide-forming activity of different strains and the level of penicillin synthesized (López-Nieto *et al.*, 1985).

The biosynthesis of ACV is catalyzed by ACV-synthetase, whose activity has been demonstrated in cell-free systems from *C. acremonium* (Banko *et al.*, 1986), and purified and partially characterized from *A. nidulans*, *C. acremonium*, and *S. clavuligerus* (Jensen *et al.*, 1990; van Liempt *et al.*, 1989; Zhang and Demain, 1990a,b). ACV-synthetase is a large enzyme with a MW ranging from 220 to 500 kDa, based on electrophoretic estimates with enzymes from various species (Aharonowitz *et al.*, 1993b). However, on the basis of the nucleotide sequence analysis of the ACV-synthetase gene, *pcbAB*, from different species a MW in the range of 405 to 426 kDa has been determined (Aharonowitz *et al.*, 1993b; Díez *et al.*, 1990; Smith *et al.*, 1990).

1.1.2.3 Conversion of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine to isopenicillin N.

The next step in the pathway is the cyclization of ACV to form isopenicillin N, a reaction catalyzed by isopenicillin N synthase (IPNS) also known as "DAOC synthase". First achieved by Fawcett *et al.* (1976) with a cell-free extract from *C. acremonium*, IPNS activity has been demonstrated in cell-free extracts of virtually all producers of classical ß-lactams (Demain and Piret, 1991), and correlates positively with the production of cephamycin (Mahro and Demain, 1987; Romero *et al.*, 1988). It is an oxygenase with requirements for dioxygen as a co-substrate, ferrous ions and ascorbate as cofactors, and DTT. The purified enzyme from different sources showed apparent Km values for ACV ranging between of 1.3 - 3.2 x 10^{-4} M (Martín and Liras, 1989), and has a broad substrate specificity (Baldwin *et al.*, 1986; Martín and Liras, 1989). The estimated MW of the fungal and *Streptomyces* spp. isozymes ranges from 26.5 to 40 kDa (Castro *et al.*, 1988; Hollander *et al.*, 1984; Jensen *et al.*, 1986; Pang *et al.*, 1984; Ramos *et al.*, 1985). IPNS genes have been cloned and sequenced from *C. acremonium* (Samson *et al.*, 1985), *P. chrysogenum* (Carr *et al.*, 1986), *A. nidulans* (Weigel *et al.*, 1988), and *Streptomyces* spp. (Leskiw *et al.*, 1988; Shiffman *et al.*, 1988; Weigel *et al.*, 1988), and shown to have significant relatedness (Ingolia and Queener, 1989; Queener, 1990).

Mechanistic studies of isopenicillin N formation, using isotopically labelled ACV, revealed that the formation of the penicillin ring system utilizes the most exothermic non-photochemical reaction available to biological systems (Baldwin and Schofield, 1992). To generate both the ß-lactam and the thiazolidine ring system of isopenicillin N, four protons are stereospecifically removed and concomitantly the dioxygen molecule is reduced to water, but none of the oxygen atoms undergoes exchange (Abraham *et al.*, 1981; Adlington *et al.*, 1982; Sawada *et al.*, 1980). The large driving force of this reaction is believed necessary to provide the means whereby the strained ring system is formed (Baldwin and Schofield, 1992).

With the formation of isopenicillin N, the biosynthetic pathway ends in those fungal species such as *Penicillium* and *Aspergillus* that produce only penicillin products from the conversion of the hydrophilic isopenicillin N into hydrophobic penicillins. This presumed two step-reaction (Martín and Liras, 1989) involves the enzyme acyl-CoA:6-aminopenicillanic acid acyltransferase (Fawcett *et al.*, 1975). Microorganisms that make cephalosporins and cephamycins do not produce this enzyme (Alvarez *et al.*, 1987), but their biosynthetic pathway is extended beyond isopenicillin N by a series of enzymes not found in hydrophobic-penicillin-producing organisms (Jensen and Demain, 1993).

1.1.2.4 Epimerization of isopenicillin N to penicillin N.

Conversion of isopenicillin N to penicillin N is carried out by an extremely labile enzyme isopenicillin N epimerase (Lübbe *et al.*, 1986), first discovered in fresh cell-free extracts of *C. acremonium* by Konomi *et al.* (1979). This enzyme catalyzes the isomerization of the L-AAA side chain to the D configuration. The somewhat more stable epimerase from streptomycetes has been studied more extensively (Jensen *et al.*, 1982b, 1983; Kovacevic *et al.*, 1990; Usui and Yu, 1989). It is a monomeric protein with an estimated MW ranging from 44-50 kDa, containing one mole of pyridoxal-5'-phosphate, no requirement for Fe^{+2}, ascorbic acid or ATP for activity, but a requirement for pyridoxal-5'-phosphate for stability (Jayatilake *et al.*, 1981; Lübbe *et al.*, 1986; Jensen *et al.*, 1983). The Km for isopenicillin N of 3×10^{-4} M was found to be lower than that for penicillin N, 7.8×10^{-4} M (Baldwin and Schofield, 1992). Because of the close physical and chemical resemblance of the substrate isopenicillin N and the product penicillin N, analysis of the epimerase activity required a procedure for the separation of the two penicillins. Two procedures developed were based on: (i) the HPLC separation of the tetraacetylglucose derivatives of the two isomers (Neuss *et al.*, 1982); and (ii) a microbiological assay making use of the differential sensitivity of *Escherichia coli* ESS 22-31 and *Micrococcus luteus* ATCC 9341 to isopenicillin N and penicillin N, respectively (Jensen *et al.*, 1983).

1.1.2.5 Conversion of penicillin N to deacetylcephalosporin C.

The reactions for the conversion of penicillin N to deacetylcephalosporin C (DAC) were established in 1970s, and consist of ring expansion and hydroxylation activities. With a cell-free system of *C. acremonium*, Demain and co-workers (Kohsaka and Demain, 1976; Yoshida *et al.*, 1978) demonstrated the conversion of penicillin N into a penicillinase-resistant material, deacetoxycephalosporin C (DAOC). That DAOC is the precursor of DAC was unequivocally demonstrated by the conversion of [3-$^{3}H_3C$]-DAOC to [3-$HO^{3}H_2C$]-DAC with cell-free extracts of *C. acremonium* (Brewer *et al.*, 1977) and *S. clavuligerus* (Turner *et al.*, 1978). Additional studies identified that this enzymic conversion, catalyzed by cell-free extracts from *C. acremonium* and *S. clavuligerus*, was optimal near pH 7.0; was stimulated by ferrous ions, ascorbate, and α-ketoglutarate; and involved dioxygen in the biosynthesis of cephalosporins (Hook *et al.*, 1979; Kupka *et al.*, 1983).

The ring expansion and hydroxylation reactions are catalyzed by DAOC synthase ("expandase") and DAOC hydroxylase (DAC synthase), respectively, both of which are α-ketoglutarate-linked dioxygenases. Although DAOC synthase has many of the characteristics of an α-ketoglutarate-linked dioxygenase (Shen *et al.*, 1984), it does not however fit the definition since the two atoms of oxygen do not end up in the products: one of the oxygen atom is incorporated into succinate during the oxidative decarboxylation of the co-substrate, α-ketoglutarate, and the other ends up in H_2O via an intermediate that is converted to DAOC (De Jong *et al.*, 1982; Pang *et al.*, 1984). In *C. acremonium*, DAOC synthase and DAOC hydroxylase activities are present as a single protein (Dotzlaf and Yeh, 1987; Kovacevic *et al.*, 1989; Samson *et al.*, 1987; Scheidegger *et al.*, 1984), with a MW of 36.5 kDa as determined by electrospray mass spectrometry (Baldwin and Schofield, 1992). In *S. clavuligerus* however, they are separate monomeric enzymes with MW of about 36 kDa and 26.2 kDa, respectively (Jensen *et al.*, 1985; Rollins *et al.*, 1988c), representing the first significant divergence to be identified between the eukaryotic and prokaryotic biosynthetic pathways to cephalosporins and cephamycins (Baldwin and Schofield, 1992).

1.1.2.6 Late reactions in cephalosporin C and cephamycin C biosynthesis.

Once the DAC stage is reached, there is a branch in the pathway. For cephalosporin biosynthesis, the enzymic acetylation of DAC by acyl-CoA : DAC *O*-acetyltransferase is the terminal reaction in the cephalosporin producing fungi, such as *C. acremonium* (Fujisawa and Kanzaki, 1975; Fujisawa *et al.*, 1975). This enzyme has an optimum pH activity of 7.0 to 7.5, and requires acetyl-CoA and Mg^{+2} for the conversion of DAC to cephamycin C.

The sequence of intermediates in the latter stage of cephamycin C biosynthesis in *S. clavuligerus* are: (i) the ATP-dependent carbamoylation of DAC by carbamoyl phosphate-3-hydroxymethylcephem-*O*-carbamoyl transferase to form carbamoyldeacetylcephalosporin C (Brewer *et al.*, 1980; Whitney *et al.*, 1972); (ii) the hydroxylation of carbamoyldeacetylcephalosporin C by another α-ketoglutarate-linked dioxygenase to make 7-α-hydroxycarbamoyldeacetylcephalosporin C, using dioxygen as the oxygen atom donor (O'Sullivan *et al.*, 1979); and finally (iii) this intermediate is methylated to cephamycin C by a methyltransferase using S-adenosylmethionine (O'Sullivan and Abraham, 1980).

3-Hydroxymethylcephem-*O*-carbamoyl transferase synthesizes a wide variety of 3-carbamoylmethoxycephems since it tolerates structural alterations around the 7-amino group of cephalosporins. This is in contrast to the highly specific DAOC hydroxylase which oxidizes the 3-methyl group of DAOC to a 3-hydroxymethyl group to form DAC. The enzyme from *S. clavuligerus* has been purified, and shown to be most active at pH 6.8 in the presence of Mg^{+2} and ATP. The enzyme activity is stabilized by phosphate anions but is inhibited by pyrophosphate anions (Brewer *et al.*, 1980; Martín and Liras, 1989).

1.1.3 Regulation of classical ß-lactam biosynthesis.

Production of classical ß-lactams occurs best under nutrient imbalance and at low growth rates (Martín and Demain, 1980), conditions that can be brought about by limitation of the carbon, nitrogen or phosphorus source (Demain and Piret, 1991; Jensen and Demain, 1993). The importance of nutrition and growth physiology in maximizing the titre of antibiotic was recognized in early studies of the penicillin fermentation with the suppressive effect on penicillin yields of unlimited growth fuelled by a rich carbon-energy source (Soltero and Johnson, 1953), and later in the production of cephamycin by actinomycetes (Aharonowitz and Demain, 1978; Cortés *et al.*, 1986; Lebrihi *et al.*, 1988a). In addition to these factors, lysine is a weak inhibitor of penicillin and cephalosporin synthesis by fungi

(Demain, 1957; Mehta *et al.*, 1979), whereas in actinomycetes lysine exerts a positive effect on the production of cephamycins (Mendelovitz and Aharonowitz, 1982). Methionine has a stimulatory effect on cephalosporin C biosynthesis in *C. acremonium* (Drew and Demain, 1973).

1.1.3.1 Nutritional factors.

Glucose supports rapid growth in most microbes and the high rate of biomass production attained when this sugar is in ample supply precludes full expression of the genes for secondary metabolism (Vining *et al.*, 1990). When glucose is not the growth substrate, other carbon sources can exert this effect. In the case of *S. clavuligerus* for instance, the range of compounds that can be used as carbon and energy sources is quite limited (Aharonowitz and Demain, 1978; Hu *et al.*, 1984; Vining *et al.*, 1987). Glycerol and maltose are good substrates for biomass production, but cause a concentration-dependent suppression of both total and specific production of cephamycin in cultures. A less suppressive carbon source is starch (Lebrihi *et al.*, 1988b).

Ammonium ion and phosphate, like glycerol, exert a negative effect on ß-lactam production in *S. clavuligerus* (Aharonowitz, 1980; Aharonowitz and Demain, 1977, 1979; Lebrihi *et al.*, 1987; Lübbe *et al.*, 1984). Other suppressive nitrogen sources are alanine and histidine, whereas asparagine, aspartate, urea and glutamate are better nitrogen sources for cephamycin production (Demain and Piret, 1991). The amino acids alanine, glutamine and glutamate have been shown to inhibit cephamycin synthesis by resting cells of *S. clavuligerus* in the absence of protein synthesis (Braña *et al.*, 1986b).

1.1.3.2 Mechanism of regulation.

The key factor in the regulation of cephamycin biosynthesis appears to be the growth rate of the culture, like the regulation of other secondary metabolites that are produced in response to low growth rate (Vining *et al.*, 1990, Demain and Piret, 1991). Lilley *et al.* (1981) reported that the production of cephamycin by *Streptomyces cattleya* was inversely related to the steady-state concentration of phosphate under growth limiting conditions imposed by a low phosphate concentration in the medium. Although Braña *et al.* (1986b) failed to observe a correlation between growth rate and cephamycin production with *S. clavuligerus* grown on different nitrogen sources, Lebrihi *et al.* (1988a) reported an inverse relationship between growth rate and cephamycin and DAOC synthase production, an effect that was not dependent on the type of the limiting-growth substrate.

The suppressive effect of nutrients is mediated by the repression or inhibition of the biosynthetic enzymes, or both. DAOC synthase was repressed by glycerol in *S. clavuligerus* and by glucose in *N. lactamdurans*, and intracellular ACV was decreased by glucose as well (Cortés *et al.*, 1986; Lebrihi *et al.*, 1988a). But no similar effect was observed for IPNS or epimerase, nor was any inhibitory effect on the enzymes by either carbon source observed. However, a relatively strong inhibitory effect was observed with glycolytic intermediates on several enzymes: ACV-synthetase by glyceraldehyde-3-phosphate, IPNS by glucose-6-phosphate, epimerase by 3-phosphoglycerate and fructose-1,6-bisphosphate, and DAOC synthase by glucose-6-phosphate, fructose-1,6-bisphosphate and fructose-2,6-bisphosphate (Lebrihi *et al.*, 1988a; Zhang and Demain, 1992a).

The repression by ammonium ion of ACV-synthetase, IPNS and DAOC synthase has been demonstrated and was found to be the major factor contributing to the negative effect of this nutrient on cephamycin biosynthesis (Braña *et al.*, 1985; Zhang *et al.*, 1989a). ACV-synthetase was the most repressible enzyme, followed by IPNS, and DAOC synthase with 75%, 70% and 50% decrease in enzyme levels, respectively, by 120 mM NH_4^+ added to the medium. The epimerase enzyme was only slightly affected, and little to no inhibitory effect was detected *in vitro* by NH_4^+ on ACV-synthetase (Zhang *et al.*, 1989a). In *S. clavuligerus*, this NH_4^+ effect is exerted to a greater extent on cephamycin formation than on the production of the co-product, clavulanic acid (Romero *et al.*, 1984). Mechanistic studies of NH_4^+ repression on cephamycin biosynthesis has indicated that this secondary metabolic process is not directly regulated by the ammonium assimilatory system (Braña *et al.*, 1986a,b).

The marked effect of phosphate on ß-lactam production was accompanied by the repression of four enzymes: ACV-synthetase, IPNS, epimerase and DAOC synthase (Lebrihi *et al.*, 1987; Lübbe *et al.*, 1984; Zhang *et al.*, 1989b). ACV-synthetase appears to be the main repression target, and IPNS the main inhibitory target by phosphate, but the latter effect was found to be due to sequestration of ferrous ions and can be reversed by their addition (Lübbe *et al.*, 1984, 1985; Zhang *et al.*, 1989b). In *N. lactamdurans*, phosphate does not have a strong effect on ß-lactam production, although a reversible and concentration-dependent inhibition of DAOC synthase was observed (Cortés *et al.*, 1986; Romero *et al.*, 1984).

As a mechanism in studying the effect of environmental factors on the regulation of antibiotic production, the possible role of oxygen is not usually investigated (Namdev *et al.*, 1992). Recently, Westlake and co-workers (Rollins *et al.*, 1988a,b, 1989a,b, 1990, 1991) examined the effect of oxygen on the production of cephamycin by *S. clavuligerus* in both rich and defined media. Unlike carbon, nitrogen and phosphorus, increased levels of oxygen enhanced the synthesis of cephamycin by *S. clavuligerus* (Rollins *et al.*, 1990, 1991), but this effect was secondary to the role of catabolite regulation of the carbon source (Rollins *et al.*, 1988a,b, 1989a,b). The timing of production of the three enzymes ACV-synthetase, IPNS and DAOC synthase was not affected by the oxygen level, though an increase in the production of DAOC synthase and a decrease in the stability of ACV-synthetase were observed under oxygen saturation (Rollins *et al.*, 1991). In addition, oxygen saturation improved the stability of IPNS in defined glycerol plus asparagine-based medium (Rollins *et al.*, 1989a).

1.2 δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine (ACV)-synthetase.

ACV-synthetase serves a key role in the biosynthesis of classical ß-lactams in both eukaryotic and prokaryotic microorganisms, for it is the initial enzyme in the biosynthetic pathways that links primary and secondary metabolism. Recently, in an article by Zhang and Demain (1992b), the progress in research on this enzyme, including the establishment of a cell-free assay system, stabilization, purification, characterization, and gene cloning was reviewed. Additional relevant information on this enzyme can be found elsewhere (Baldwin and Schofield, 1992; Jensen and Demain, 1993; Zhang and Demain, 1991). Thus in the following account, emphasis is placed mainly on the mechanistic studies, stability and biochemical properties of ACV-synthetase.

1.2.1 Peptide synthetase and the thiotemplate reaction mechanism.

ACV-synthetase belongs to a class of enzymes known as non-ribosomal peptide synthetases (Aharonowitz *et al.*, 1993b). Some of the most studied enzymes in this class are the *Bacillus* gramicidin S synthetases (Gutiérrez *et al.*, 1991; Lipmann, 1980; Vandamme, 1981; Vater, 1990), tyrocidine synthetase (Hori *et al.*, 1978; Kleinkauf and Koischwitz, 1980; Mittenhuber *et al.*, 1989) and bacitracin synthetase (Ishihara *et al.*, 1982; Kleinkauf and von Döhren, 1988), and the *Fusarium* enniatin synthetase (Zocher and Kleinkauf, 1978; Zocher *et al.*, 1982). These are characteristically large enzymes with relatively broad substrate specificity, which are extremely labile (Billich and Zocher, 1988; Kleinkauf and von Döhren, 1983a; Vandamme, 1990; Vandamme *et al.*, 1982), and only transitorily synthesized during the growth phase of the producing microorganisms (Kleinkauf and von Döhren, 1983a,b; Vandamme, 1990). It is therefore difficult to prepare large quantities of these enzymes.

The biosynthetic process catalyzed by the peptide synthetases has been called a "protein template mechanism" (Lipmann, 1971), a "thiotemplate mechanism" (Laland and Zimmer, 1973), or a "multienzyme thiotemplate mechanism" (Kurahashi, 1981). The reactions involved are amino acid activation, enzyme aminoacylation, amino acid racemization, and peptide bond formation with transport of peptides by 4'-phosphopantetheine (Kleinkauf and von Döhren, 1983a). Information from a number of studies indicates that ACV-synthetase also displays similar properties to other peptide synthetases (Aharonowitz *et al.*, 1993b; Baldwin and Schofield, 1992; Zhang and Demain, 1992b). *In vivo* synthesis of ACV by *P. chrysogenum* was shown to be stimulated by the addition of protein synthesis inhibitors cycloheximide and anisomicin (López *et al.*, 1985), consistent with the suggestion that it is biosynthesized by a non-ribosomal process (Fawcett and Abraham, 1975).

In addition to the evidence on the lability and large size of ACV-synthetase, firm evidence for the proposal that it is a non-ribosomal peptide synthetase comes from mechanistic studies on the enzyme from *A. nidulans* (van Liempt *et al.*, 1989), and establishment of the presence of phosphopantetheine in the *C. acremonium* and *S. clavuligerus* enzymes (Baldwin *et al.*, 1990, 1991; Gutiérrez *et al.*, 1991a). As summarized by Baldwin and Schofield (1992), the highly purified ACV-synthetase:

(i) catalyzed the formation of ACV from the L-enantiomers of the constituent amino acids in the presence of ATP, Mg^{+2} and dithioerythritol, a reaction that involved also the epimerization of L-valine to the D-configuration;

(ii) catalyzed ATP-pyrophosphate exchange in the presence of each of the three amino acids independently, consistent with a mechanism involving formation of the amino acid acyl adenylates, and with a single multienzyme being responsible for the formation of the tripeptide;

(iii) reacted with and bound [^{14}C]-valine in an ATP-dependent reaction in the presence of appropriate cofactors, but in the absence of the other two substrate amino acids;

(iv) released valine from the enzyme by the addition of performic acid, but not by formic acid, indirect evidence of a mechanism by which the valine is bound to a sulfhydryl group of the enzyme in a thioester linkage; and finally

(v) contained one mole and three moles of phosphopantetheine, as determined from two independent studies (Baldwin *et al.*, 1991; Gutiérrez *et al.*, 1991a).

On the basis of these findings, a mechanistic scheme for the biosynthesis of ACV has been proposed and is depicted in Figure 1.3, in which each of the amino acids is activated as the corresponding acyl adenylate before binding to the multienzyme by a thioester linkage (Kleinkauf and von Döhren, 1990b; Zhang and Demain, 1992b). This reaction starts with the activation of L-AAA and L-cysteine, followed by the formation of the dipeptide δ-(L-α-aminoadipyl)-L-cysteine (Ramos *et al.*, 1985). The last step of this reaction involves the conversion of the enzyme-bound dipeptide into ACV by the reaction of L-valine with an activated form of the dipeptide (Fawcett *et al.*, 1976). It is envisaged that the valinyl residue would be epimerized at the thioester stage (either before or after the formation of the tripeptide). Intermediate peptidyl thioesters are transported to the next acyl residue by the 4'-phosphopantetheinate arm, where the thioester bond is cleaved from the enzyme by a thioesterase.

1.2.2 Production of ACV-synthetase.

Studies on ß-lactam production have shown a good correlation between the activity of ACV-synthetase and the level of penicillin and cephalosporin synthesized (Martín and Liras 1989). From early studies on ß-lactam production, the best time to harvest the mycelium to observe ACV-producing activity was determined to be during the early phase of cephalosporin biosynthesis (Martín and Liras 1989). However, the timing of this early phase of antibiotic biosynthesis may vary with a number of factors, including the type of culture, the type of inoculum, and the growth conditions. Because of the complexity of this system, predictions of the time for maximum yield are quite difficult to make without a good understanding of the system.

Fortunately, much is known about the effects of environmental factors on the regulation of formation and activity of cephalosporin biosynthetic enzymes, including

Figure 1.3 Mechanistic scheme for the biosynthesis of ACV.

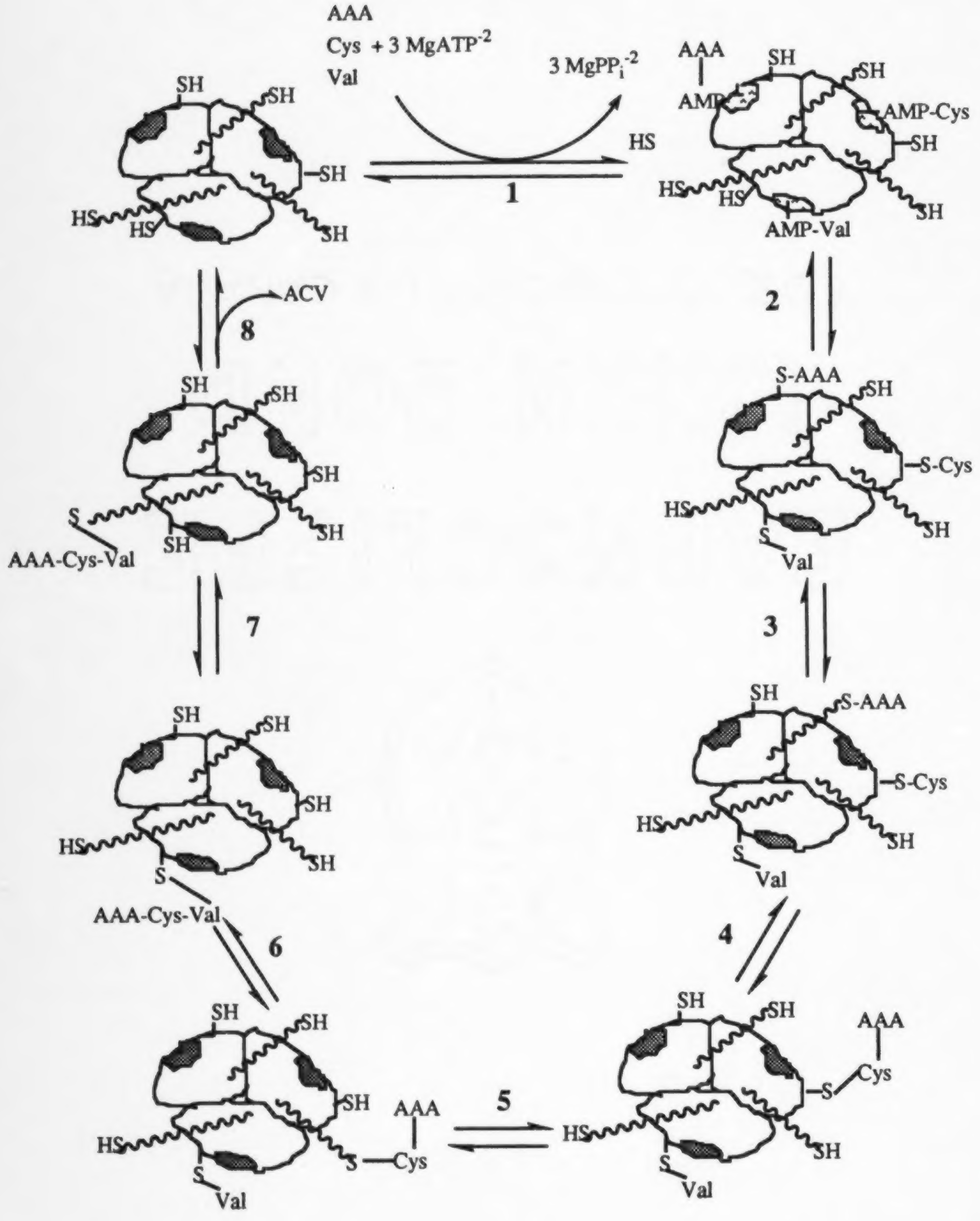
The hypothetical reaction mechanism of ACV-synthetase showing the three binding sites (shaded) for each individual amino acids (L-AAA, L-Cys and L-Val), and each containing a 4'-phosphopantetheine (〰SH).

1. MgATP^{-2}-dependent amino acid carboxyl activation as adenylates and enzyme aminoacylation.

2. Thioesterification and epimerization of L-Val to D-Val.

3, 4, 5 and **6.** Sequential steps for peptide bond formation involving the transport of the peptide by 4'-phosphopantetheine.

7 and **8.** Release of ACV.


AAA
Cys + 3 MgATP^{-2}
Val
3 MgPP$_i^{-2}$
1
2
3
4
5
6
7
8
ACV
SH
HS
AMP
AMP-Cys
AMP-Val
S-AAA
S-Cys
S
Val
AAA
Cys
AAA-Cys-Val

ACV-synthetase (Zhang and Demain, 1991, 1992b), which has allowed the modeling of the kinetics of production of this enzyme (Malmberg and Hu, 1991; Namdev *et al.*, 1992). Analysis of the time course of appearance of the biosynthetic enzymes indicates that their activities follow a pattern consistent with a burst of transcription followed by gradual inactivation. The production and stability of ACV-synthetase is influenced by catabolite regulation (Jensen and Demain, 1993) and oxygen supply (Rollins *et al.*, 1991), and highest enzyme yields are achieved during fermentations in complex media under uncontrolled dissolved oxygen level (Namdev *et al.*, 1992). However, the specific activity of ACV-synthetase under typical growth conditions remains the lowest, at 1 to 10%, of the activity of the other three enzymes (IPNS, DAOC-synthase, and DAOC hydroxylase) immediately downstream in the pathway, as revealed from the comparison of these biosynthetic enzymes in crude cell-free extracts of *C. acremonium* and *S. clavuligerus* (Namdev *et al.*, 1992; Zhang and Demain, 1992a). Therefore, the conditions for maximizing yields of ACV-synthetase must take into consideration the effects of environmental factors on enzyme production and stability, in combination with genetic manipulation of the system (Jensen and Demain, 1993).

1.2.3 Characteristics of ACV-synthetase.

The development of a reliable cell-free assay, coupled with a partial stabilization of ACV-synthetase with glycerol by Banko *et al.* (1986, 1987) have facilitated studies of the stability and biochemical properties of this enzyme *in vitro*.

1.2.3.1 Stability properties.

Proteins are marginally stable under physiological conditions, with a free energy of denaturation of only 5 to 20 kcal/mol (Pace, 1990; Privalov and Khechinashvili, 1974). This is because the stabilizing free energy, arising from interresidue interactions in a globular structure, is largely canceled by loss of entropy that arises from the compactness of the protein (Arakawa *et al.*, 1991). A broad spectrum of solvent additives has been shown to affect the stability of proteins, under widely different conditions. In solution, sugars, polyols, and certain amino acids and salts are known to be protein stabilizers (Arakawa and Timasheff, 1983, 1985; Back *et al.*, 1979; Gerlsma, 1968; von Hippel and Wong, 1962, 1965), whereas hydrophobic organic compounds, chaotropic salts, urea and guanidine hydrochloride are known to be protein destabilizers (Gerlsma, 1968; Schellman, 1978; Tanford, 1968; Timasheff and Inoue, 1968). Of importance, is a class of compounds, such as polyethylene glycol and 2-methyl-2,4-pentanediol, that destabilize proteins at high temperature but stabilize them during freezing-thawing (Arakawa *et al.*, 1991).

Mechanistically, these effects can be explained by the preferential interaction of the co-solvents with the proteins, where the structure-stabilizing compounds are preferentially excluded from surface of the proteins and the destabilizers preferentially bind to the proteins. The addition of stabilizer increases the chemical potential of the protein and thus the free energy of the system, a condition that is thermodynamically unfavorable and that ultimately results in stabilization of the native conformation (Arakawa *et al.*, 1991; Gekko and Ito, 1990). Exclusion of a wide variety of compounds is determined by their effect on the surface tension of water. Co-solvents perturb the cohesive force of water, hence decreasing or increasing its surface tension, which results in either an excess or a deficiency of the co-solvent in the protein surface layer (Arakawa *et al.*, 1991; Gibbs, 1978). The complex effects of polyethylene glycol and 2-methyl-2,4-pentanediol can be accounted for by their preferential exclusion from native proteins determined at room temperature and from their hydrophobic character, which depends on temperature.

Enzyme inactivation mechanisms that have been invoked for peptide synthetases are (i) increased oxygen tension and intracellular redox state of the culture (Agathos and Demain, 1986; Friebel and Demain, 1977a,b), and (ii) induction of synthetase specific proteinases (Gaucher *et al.*, 1982; Lam *et al.*, 1988) during the idiophase. Crude ACV-synthetase from *C. acremonium* has half-lives of 10 h and 2 h at 4°C and 25°C, respectively (Zhang and Demain, 1990a); and the *S. clavuligerus* enzyme has a half-life of 25 h at 4°C (Zhang and Demain, 1990b).

Enhancement of ACV-synthetase stability has been demonstrated with glycerol (Banko *et al.*, 1986) and other additives, including those commonly used to stabilize other peptide synthetases. Recently, it was reported that the incorporation of the cofactors Mg^{+2} , DTT (5 mM), together with crude *C. acremonium* ACV-synthetase, obtained in the form of a narrow range ammonium sulfate fraction of the extract, resulted in an increase in the half-life this enzyme from 10 h to about 100 h at 4°C and 18 h at 25°C (Zhang and Demain, 1990a, 1992b). Similarly, the crude *S. clavuligerus* enzyme stability was increased by three-fold at 4°C. Further addition of L-valine and ATP to the stabilization cocktail resulted in a dramatic improvement of the stability of the purified *S. clavuligerus* enzyme, where 50% retention of initial activity was observed after 15 days at 4°C.

Improved stability of ACV-synthetase by the reducing agent DTT has implicated the oxidation of enzyme sulfhydryl groups as a factor in instability of the enzyme, as has been the case with other peptide synthetases, such as gramicidin S synthetase. Recently, Rollins *et al.* (1991) have studied the effect of dissolved oxygen level on ACV-synthetase synthesis and activity during growth of *S. clavuligerus*, and found that the maintenance of high dissolved oxygen levels throughout the fermentation did not affect the formation of this enzyme but reduced its *in vivo* stability. Maintaining a reduced environment in the vicinity of ACV-synthetase both *in vivo* and *in vitro* appears to be critical for ACV-synthetase stability.

1.2.3.2 Biochemical properties.

The kinetics of ACV formation from substrate amino acids, ATP and Mg^{+2} have been studied with partially purified and purified ACV-synthetase, and are shown in Table 1.1. The Michaelis parameters determined for the crude *C. acremonium* enzyme (Banko *et*

Table 1.1 Michaelis constants for ACV-synthetase substrates

Organism	L-AAA	L-cysteine	L-valine	Reference
C. acremonium (crude)	1.7×10^{-4} M	2.6×10^{-5} M	3.4×10^{-4} M	Banko *et al.*, 1987
S. clavuligerus (crude)	5.6×10^{-4} M	7.0×10^{-5} M	1.1×10^{-3} M	Jensen *et al.*, 1988
S. clavuligerus (pure)	6.3×10^{-4} M	1.2×10^{-4} M	3.0×10^{-4} M	Zhang *et al.*, 1992

al., 1987) were three-fold lower than those reported for the crude *S. clavuligerus* enzyme (Jensen *et al.*, 1988). Recently, similar studies were carried out with purified *S. clavuligerus* ACV-synthetase, and the apparent Km values were again different from those reported previously with the crude enzyme (Zhang *et al.*, 1992).

One of the characteristics of peptide synthetases is their broad substrate specificity. Replacement of natural substrates has been investigated with ACV-synthetase for the synthesis of ACV analogues, as a means to develop novel antibiotics (Baldwin and Schofield, 1992). The enzymes from *C. acremonium* and *S. clavuligerus* were shown to use at various catalytic rates L-carboxymethylcysteine for L-AAA, L-*allo*-isoleucine or L-α-aminobutyrate for valine (Baldwin *et al.*, 1990; Banko *et al.*, 1987; Jensen *et al.*, 1988). The enzyme from *S. clavuligerus* displayed strict specificity for L-AAA analogues. Changes in chain length, functional groups or steric configuration were not tolerated. On the other hand, it displayed relatively loose specificity for L-valine analogues and some tolerance for L-cysteine analogues (Zhang *et al.*, 1992).

1.3 Antibiotic production by immobilized biocatalysts.

There are three ways of producing known and novel antibiotics: chemical processes, biological processes or a combination of the two approaches. Chemical routes are known for the total synthesis of many important antibiotics, including ß-lactams (Colvin, 1992; Cooper, 1992). However, despite the existence of these chemical routes, only a few compounds such as chloramphenicol, thienamycin, cellocidin, which are relatively simple antibiotics, are produced industrially by chemical synthesis (Vandamme, 1990). Most antibiotics are very complex biological molecules, whose synthesis involves complex pathways and multi-step enzymic reactions, virtually excluding large scale chemical production from an economic point of view. They are therefore produced by microorganisms, and in turn several new compounds with superior therapeutic properties are derived from such fermentation products by chemical or single step enzymic conversions (Savidge, 1984; Vandamme, 1983, 1984).

Apart from conventional batch fermentation or bioconversion processes, novel ways for producing antibiotic compounds are via immobilized biocatalyst-based processes, which are centered around three areas. These include the multi-step total or partial synthesis using immobilized biocatalysts, antibiotic fermentation using immobilized cells, and antibiotic bioconversions with immobilized biocatalysts (Vandamme, 1990). The latter has met with great success industrially by replacing laborious or uneconomical chemical processes. However, immobilized biocatalysts have not yet found general applicability in the production areas involving complex pathways and multi-step enzymic reactions, areas in which fermentation will probably never be displaced from its position in industrial biotechnology as the leading biocatalyst process (Scouten, 1983).

This section will focus on the use of immobilized enzymes for partial or total production of ß-lactam antibiotics, but excluding processes that are already operational on a large scale for example the production of 6-aminopenicillanic acid (6-APA) and 7-aminocephalosporanic acid by enzymes not involved in the biosynthetic pathway. Review articles on this subject can be found elsewhere (Lowe, 1985; Mahajan, 1984; Poulsen, 1984; Savidge, 1984; Vandamme, 1980, 1983, 1990). Because of the similarity between peptide synthetases and ACV-synthetase, information on peptide antibiotic biosynthetic processes involving immobilized multifunctional peptide synthetases will be considered. It may, however, be appropriate first to give a brief general introduction of different enzyme immobilization techniques that have been used over the years. A comprehensive treatment of these methods can be found in a number of review articles and books (Bernath and Venkatasubramanian, 1986; Kennedy and Cabral, 1983, 1987; Mosbach, 1976, 1987; Trevan, 1980).

1.3.1 Immobilized enzyme techniques.

Immobilized enzymes are defined as enzymes which are in the state permitting their continuous or intermittent use over an extended period of time (Poulsen, 1983). They are usually classified by the method of binding or localization which allows reuse (Zaborsky, 1973). Immobilized enzymes are also classified on the basis of the nature of the support and the type of coupling reaction (Kennedy and Cabral, 1983). The latter classification consists of two major groupings. The first is the "insoluble enzyme methods", which result in the insolubilization of enzyme. They consist of binding methods: crosslinking and carrier binding (physical adsorption, ionic binding, metal binding or covalent binding); and entrapping methods: gel entrapping, fiber entrapping and microencapsulation. The second grouping is the "soluble enzyme methods", resulting in the physical confinement of soluble enzymes in semipermeable membranes or hollow bore films.

Almost every possible immobilization procedure has been applied to the penicillin acylase enzyme or its producing organisms (Savidge, 1984; Vandamme, 1980, 1983). This enzyme carries out the hydrolytic reaction of penicillins into 6-APA, a reaction that continues to serve as an important system for the design of new immobilization techniques and reactors. A few examples are immobilization by adsorption onto bentonite or diatomaceous earth (Ryu *et al.*, 1971), ionic bonding onto various ion-exchange resins, crosslinking with glutaraldehyde (Savidge and Powell, 1977), and covalent attachment with the cyanogen bromide activation of carriers such as cellulose or Sepharose 4B (Lagerlöf *et al.*, 1976).

1.3.2 Production of peptide antibiotic by immobilized enzymes.

Immobilized enzymes have to be superior to fermentation methods in cost, quality, or novelty of the product before these processes could displace fermentation and other solution methods, most of which are relatively simple (Scouten, 1983). This is shown by the relatively small number of immobilized enzyme systems that have been described as an industrial successes (Poulsen, 1984). All these processes pertain either to carbohydrate processing, to penicillin and cephalosporin processing, or to separation of amino acids. Although universally applied, conventional fermentations processes display several drawbacks that need to be improved. First, the synthesis of antibiotic, as a secondary metabolite, is usually delayed until growth declines; second, the "antibiotic synthetases" are unstable and rapidly inactivated; third, the conversion of substrate into antibiotic is inefficient with only 10% conversion into penicillin; and fourth, the degeneration of antibiotic producing strains presents a universal problem (Vandamme, 1990). Several attempts have been made to replace fermentation or cellular synthesis by acellular processes. The concept in such processes aims at the ultimate use of isolated (and immobilized) enzymes to carry out the sequential reactions for the total synthesis of an antibiotic from precursors.

1.3.2.1 Total enzymic synthesis.

Successful total enzymic synthesis of antibiotics has been reported with several peptide synthetases. The most studied processes are the biosynthesis of oligopeptide antibiotics, including gramicidin S, tyrocidine, bacitracin, and enniatins. By virtue of their multi-functional nature, these enzymes are capable of carrying out in a multi-step reaction the total synthesis of the peptide antibiotic (Kleinkauf and von Döhren, 1983a,b). The process that is best understood is the synthesis of the cyclic decapeptide, gramicidin S, produced by certain *Bacillus brevis* strains (Vandamme, 1990; Vater, 1989). A two-enzyme complex, gramicidin S synthetases 1 and 2 isolated from the high gramicidin S producing strain of *B. brevis*, has been shown to produce gram quantities of gramicidin S *in vitro* (Demain and Wang, 1976). When co-immobilized with an ATP-regenerating system in a continuous process, these enzymes also produced sufficient quantities of gramicidin S, necessary for the chemical and biological analyses to prove identity of this compound (Tramper, 1983; Trevan, 1980).

Enniatin synthetase, a 250-kDa monomeric enzyme from the fungus *Fusarium oxysporum*, has also been immobilized and shown to synthesize different types of the depsipeptide antibiotic enniatin. Madry *et al.* (1984) reported on the immobilization by adsorption of the partially purified enzyme with 45% retention of original activity and an operational half-life of 15 h. Although the product yield was low, the immobilized enzyme system was more selective and displayed a broader substrate specificity than the cellular system as source of enzymes, in synthesizing not only several enniatin homologues but also non-natural depsipeptides. The lower stability of the adsorbed enzyme in comparison to the soluble enzyme was overcome by the covalent immobilization of the enzyme to N-hydroxysuccinimide activated agarose (Siegbahn *et al.*, 1985).

1.3.2.2 Partial enzymic synthesis.

Unlike oligopeptide antibiotics that are synthesized in a single enzyme multi-step reaction process, ß-lactam antibiotics are biosynthesized by separate enzymes in sequential multi-reaction process. The total synthesis of cephamycin and cephalosporin *in vitro* is only possible if all enzymes, substrates and cofactors involved in the pathway are known and available – conditions that have been met with recent intensive studies of biosynthetic pathways of ß-lactam producing organisms (see Figure 1.2) (Baldwin and Schofield, 1992; Demain and Piret, 1991; Jensen and Demain, 1993). The next logical step is to attempt the total synthesis of these antibiotics with immobilized enzymes.

Prior to the current advances in the knowledge of the ß-lactam antibiotic biosynthetic pathways, Jensen *et al.* (1982b, 1984) reported on the ionic binding onto DEAE-Trisacryl resin of the four soluble enzymes, from IPNS to DAOC hydroxylase, present in the cell-free extract of *S. clavuligerus*. This system was shown to carry out the continuous multi-step conversion of the natural intermediate ACV into a mixture of penicillin and cephalosporin ß-lactam antibiotics (Jensen *et al.*, 1984). Two of the drawbacks of this immobilized biosynthetic machinery were its limited stability upon repeated use and the high cost of the chemically synthesized precursors, ACV and its analogues. Progress in the stabilization of ACV-synthetase allowed the immobilization of this enzyme from *S. clavuligerus* onto DEAE-Trisacryl and its repeated use in the synthesis of ACV and its analogues (Jensen *et al.*, 1989). This system also needed improvements with respect to stability and productivity. Attempts for the total enzymic ß-lactam antibiotic biosynthesis from the amino acids failed due to the inhibitory effects of free L-AAA and cysteine on IPNS (Jensen and Westlake, 1989).

A recurring theme with immobilized or soluble enzyme systems is the relatively broader substrate specificity they display in comparison to the whole-cell system of conventional fermentation processes. IPNS, which does not tolerate large changes in the ACV molecule (Martín and Liras, 1989), has however the ability to cyclize a variety of ACV analogues, such as phenylacetyl-L-cysteinyl-D-valine, and directly synthesize some existing and novel ß-lactams (Castro *et al.*, 1986; Luengo *et al.*, 1986a). Similarly, immobilized *P. chrysogenum* acyl CoA:6-APA acyltransferase could be used for *in vitro* synthesis of different penicillins by direct acylation of the penicillin nucleus, 6-APA (Luengo *et al.*, 1986b; Vandamme, 1990).

The potential applications of total or partial enzymic synthesis can be deduced from the studies described above. These include the improvement of current antibiotic production, the production of biosynthetic intermediates for conversion into new antibiotics, the total synthesis of novel antibiotics in the absence of cellular permeability barriers or toxicity, and the synthesis of other complex biochemicals (Vandamme, 1990). One of the key parameters determining the feasibility of a biotechnological process is the combination of efficiency and stability of the biocatalyst (Chibata, 1978; Zaborsky, 1973). It has been proposed that immobilization of an enzyme very often results in a greatly increased resistance to a variety of denaturation factors (Monsan and Combes, 1988). This is particularly important for immobilized ß-lactam biosynthetic enzymes, whose stability *in vitro* needs to be greatly improved. In addition, with the advent of genetic engineering techniques which make the overproduction of almost any enzyme achievable, isolated enzymes will no doubt play increasingly important roles as biocatalysts for organic synthesis (Jensen and Westlake, 1989).

1.4 High-level production and refolding of recombinant proteins.

In the past few years heterologous gene expression has become a powerful technology for the production of a large number of useful biologicals in microorganisms, that were previously either unavailable or only obtainable in minute quantities (Glick and Whitney, 1987). This recombinant DNA technology has also found wide application in the expression of new genes or cDNA to verify the isolation of correct sequences, and for the preparation of wild-type or mutagenized proteins for structure-function experiments. After the initial successes in cloning foreign genes into industrially useful hosts, and expressing the desired polypeptide chain, the problem of the product appeared to be simply that of scale up. But, despite numerous successful examples, difficulty soon emerged in recovering the active, properly-folded proteins (Simons *et al.*, 1984; Taylor *et al.*, 1986). The major problem encountered was the accumulation of the expressed polypeptide chains as aggregates of denatured proteins, known as inclusion bodies (Mitraki and King, 1989).

In the following sections, certain general aspects pertaining to overproduction and recovery of proteins of relevance to this thesis are presented. Extensive coverage of heterologous gene expression can be found in a number of review articles and books (Berger and Kimmel, 1987; Goeddel, 1990; Reznikoff and Gold, 1986; Shatzman and Rosenberg, 1987; Wu, 1989).

1.4.1 Advantages and disadvantages of *E. coli*-based expression systems.

Every expression system is composed of two elements: the DNA directing the protein synthesis and the host. Over the years, a number of hosts have been developed for heterologous protein production. Among these, the *Escherichia coli*, *Bacillus subtilis*, yeast, and mammalian cell systems are considered the four major expression systems (Balbas and Bolivar, 1990). Historically however, *E. coli* has remained the major host for the expression of heterologous proteins. Generally, any protein can be produced in *E. coli* as long as it is not too small, too large, too hydrophobic, and does not contain too many cysteine residues. The preferential use of *E. coli*-based expression systems results from the advanced knowledge of its genetics and physiology, its rapid growth rate and low cost of cultivation. The major drawbacks of *E. coli* as a host for the overproduction of proteins are its limited capacity to secrete proteins and its inability to exert certain posttranslational modifications of the protein, often resulting in wrong protein folding.

1.4.2 Factors affecting the expression of foreign proteins in *E. coli*.

The increased knowledge of the rate-limiting steps in the synthesis and degradation of mRNA and proteins has revealed a range of factors that affect the expression of foreign proteins in *E. coli*. Plasmid-based systems require a well-characterized origin of replication, a selection marker for plasmid propagation and maintenance, and elements that ensure transcription initiation and efficient translation (Balbas and Bolivar, 1990). The factors that affect the overall product yield of a plasmid-directed expression system in *E. coli* may then be grouped into three categories consisting of the plasmid-related elements, mRNA-related elements, and protein-related elements.

1.4.2.1 Plasmid-related factors.

Plasmids are characterized by a number of features essential for their successful use as expression vectors. Balbas and Bolivar (1990) listed a number of functional features of commonly used plasmid replicons such as pBR322 (Filutowicz *et al.*, 1985; Polisky, 1986; Scott, 1984), which include: the incompatibility group, copy number, mobilization, replication aspects, host range and host functions required for replication. Of these, replication and copy number of the plasmid are the most important features (Balbas and Bolivar, 1990). High-copy-number plasmids are useful for the production of proteins whose expression at high levels does not perturb the cell. But in practice, maximal levels of gene expression may occur when the plasmid copy number is relatively low (Aiba *et al.*, 1982; De Boer *et al.*, 1983). Also important is the stability of the plasmid itself, which is determined by the replicon region, the growth conditions, the physiology of the host strain employed and the genes it contains. The two different types of plasmid instability that have been identified are segregational instability and structural instability (Balbas and Bolivar, 1990; Ensley, 1985). Several approaches have been developed to diminish the effects of segregational instability, and the most common method involves the inclusion of antibiotics which select for the presence of plasmids carrying the appropriate antibiotic resistance genes (Ausubel *et al.*, 1987; Balbas *et al.*, 1986).

1.4.2.2 mRNA-related factors.

The overall yield of plasmid-directed expression systems in *E. coli* depends also on factors affecting the production and stability of mRNA. The cellular mRNA levels are affected by the rate of transcription initiation, elongation and termination, and mRNA decay or degradation. Transcription initiation is a rate-limiting process for mRNA synthesis. In *E. coli*, the frequency of transcription initiation is programmed by the promoter sequence, which can be modulated by the interaction of one or more regulatory proteins with specific sequences in the vicinity of the promoter (Balbas and Bolivar, 1990). Therefore, for efficient transcription initiation, the expression vector must contain a strong, regulatable promoter sequence such as *trp, lac*UV5, *lpp*, λP_L, or λP_R (Balbas *et al.*, 1986; Brosius, 1988; Denhardt and Colasanti, 1988). The most widely used induction methods include heat (λP_L), chemical inducers (*trp, lac,* λP_L), and nutrient starvation (*trp, pho*A) (Balbas and Bolivar, 1990).

Generally, high basal expression levels or failure to regulate the expression of strong promoters is not a desirable characteristic for achieving good product yield, which often leads to slow growth of the cells, accumulation of plasmid deletions, accumulation of cells lacking the plasmid, or death of the cells (Balbas and Bolivar, 1990; Yansura and Henner, 1990). To partially overcome this problem, runaway replicating and amplifiable-copy-number plasmids have been used (Bittner and Vapnek, 1981; Panayotatos, 1988; Remaut *et al.*, 1983). However, there are drawbacks that limit the usefulness of these approaches, which include the repression of the system that is likely to be abolished by titration of repressors, the amplification of the plasmid-encoded accompanying genes that may compromise cellular functions and complicate purification of the desired product, and the possibility of interference between elevated transcription and extensive replication.

In the case of plasmids carrying P_L, as in bacterial hosts containing an integrated copy of a portion of the phage genome (bacterial lysogens) (Shatzman and Rosenberg, 1987), transcription is controlled by the phage repressor protein (*c*I), a product synthesized continuously and regulated autogenously by the lysogen (Ptashne *et al.*, 1976). When the lysogen carries a temperature-sensitive repressor (*c*I857) (Sussman and Jacob, 1962), P_L-directed transcription can be controlled by the temperature of the culture and hence transcription can be activated at any time by a temperature shift. The disadvantage of this mode of induction is the generalized heat-shock response in *E. coli*, that results also in the induction of proteinases (Baker *et al.*, 1984; Goff *et al.*, 1984; Neidhardt *et al.*, 1984). Alternatively, a lysogen carrying a wild-type repressor gene (*c*I^+) can also be induced to synthesize the desired gene product, using a DNA-damaging agent such as nalidixic acid. This mode of induction leads to an SOS response by the host (Little and Mount, 1982), a cellular state different from the heat induced state. Such variations can lead to significant differences in gene product accumulation.

1.4.2.3 Protein-related factors.

The assembly of amino acids into a polypeptide chain is an mRNA-directed multi-component process, which involves base-pairing among three different RNA species, and interactions with the ribosomal proteins. Several mRNA elements have been identified that condition the initiation of translation (Gold *et al.*, 1984; Stormo *et al.*, 1982; Stranssens *et al.*, 1986). Among these the initiation codon, the sequence and distance of the Shine-Dalgarno or ribosome binding site sequence are of paramount importance. Two other processes that ensure the synthesis of the right product are elongation of the nascent polypeptide and the peptide chain liberation. In *E. coli*, weakly expressed genes are characterized by occurrence of codons recognized by rare tRNA species, a reflection of the pattern of nonrandom codon usage (De Boer and Kastelein, 1986; Bulmer, 1987). The last important parameter pertaining to protein yield is protein degradation, a carefully regulated process that has a deep influence on the degree of protein accumulation in *E. coli* (Goldberg and Goff, 1986). Some strategies have been used to overcome selective degradation of cloned proteins (Randall and Hardy, 1984; Shatzman and Rosenberg, 1990).

1.4.3 Expression of recombinant proteins in *E. coli.*

Expression of cloned eukaryotic and prokaryotic genes in *E. coli* has become a widely used means of obtaining large amounts of biologically important proteins. Two strategies have been used to produce heterologous gene products in *E. coli*. The first strategy involves the co-translational secretion of recombinant protein in a native form, and the second strategy is based on the production of the heterologous protein inside the cell, followed by its recovery from the cell (Stader and Silhavy, 1990).

E. coli can be genetically engineered to secrete heterologous gene products in the culture supernatants. This ability to secrete heterologous proteins is desirable for a number of reasons. It permits the development of an industrial continuous culture fermentation process, achieving higher yields and facilitating the purification of the product (Stader and Silhavy, 1990). The products can be recovered with the correct primary amino acid structure, lacking the additional initiating methionine. Because the periplasmic or extracellular environment of the cell is less reduced and free of harmful cytoplasmic proteinases, the yield of correctly folded disulfide bond-containing proteins could be improved while avoiding the proteinolytic degradation of the product. The strategy of co-translational secretion of protein to an oxidizing environment, either the periplasm or the extracellular medium, has successfully worked in *E. coli* for a number of proteins including human proteins of clinical importance (Hsiung *et al.*, 1986; Nagahari *et al.*, 1985; Takahara *et al.*, 1988). The major problems that have been encountered are: (i) inefficiency or incomplete translocation of the molecule across the inner membrane, (ii) insufficient capacity of the localization machinery, (iii) proteinolytic degradation, (iv) anomalous folding and absent or incorrect post-translational modification, (v) and cell death from overloading of the export machinery due to inefficient secretion or toxicity of the foreign protein.

A common alternative strategy for production of heterologous proteins involves intracellular expression and maturation of the product. Frequently however, the overexpressed proteins are inactive because the polypeptide chains do not readily adopt their native conformation in *E. coli*, and may then accumulate in the cytoplasm in the form of insoluble inclusion bodies (Kane and Hartley, 1988; Kohno *et al.*, 1990).

1.4.3.1 Mechanism of inclusion body formation.

Initially inclusion body formation was attributed to the generation of an incorrectly transcribed or translated sequence or to secondary damage. But the subsequent recovery of biologically active proteins from such materials, after unfolding and refolding the polypeptide *in vitro*, established that the problem in the inclusion body chain was one of conformation, and not of sequence or modification (Mitraki and King, 1989). It is now known as a general phenomenon of bacterial expression, involving secreted as well as intracellularly expressed proteins (Nilsson and Anderson, 1991). *In vitro* studies of protein unfolding and refolding indicate that aggregation occurs from incorrect, but specific association of partially folded intermediates in the folding pathway, not from native or fully unfolded proteins (Goldberg, 1985; London *et al.*, 1974). These intermediate species are now known as the the "collapsed forms" (Kim and Baldwin, 1990) or "molten globules" (Dolgikh *et al.*, 1981; Goto *et al.*, 1990; Ogushi and Wada, 1983, Ptitsyn *et al.*, 1990), characterized by a structure that is compact, with a native secondary structure but a poorly defined tertiary structure.

The failure to fold properly *in vivo* results from a number of factors: lack of cellular compartmentation causing the protein to be produced in a reducing environment, lack of mammalian post-translational modifying enzymes, lack of molecular chaperones, proper folding enzymes and thioredoxin during production, and the instability of the native tertiary structure (Schein, 1989). Less plausible reasons that have been proposed are the recognition of the recombinant protein as foreign by *E. coli* and the lack of temporal control between a high production rate that greatly exceeds the folding rate. These, in addition to the popular view that proteins are synthesized to levels above their solubility, or that some other form of precipitation of the native protein is taking place (Kane and Hartley, 1988).

The lack of appropriate conditions in the host has been primarily implicated in the formation of eukaryotic protein inclusion bodies in *E. coli*. Many therapeutically useful heterologous proteins synthesized in *E. coli* are secreted molecules in their native state, requiring glycosylation and disulfide-crosslinking for both stability and solubility. Such stabilizing influences are unavailable in the bacterial cytoplasm (Schein, 1989; Mitraki and King, 1989), and the more reduced *E. coli* cytoplasm does not seem to be favorable to disulfide bond formation. In addition, the pH and ionic strength of the *E. coli* cell may not be suitable for the solubilization of certain eukaryotic proteins. Insulin for instance has a limited solubility in bacterial cytoplasm at pH 7.8 (Dinnbier *et al.*, 1988). Yet in the natural host, insulin can be stored in a soluble form in acidic secretory granules, although the pH within the endoplasmic reticulum and the mass of the eukaryotic cytoplasm is neutral.

Other factors that may lead to increased aggregation are the protein sequence, slow refolding rates, and growth temperature. A positive correlation has been reported between stretches of hydrophobic residues, average protein charge, and the fraction of turn-forming residues, and tendency to form inclusion bodies (Blum *et al.*, 1992; Schein, 1989). Some mammalian enzymes are extremely hydrophobic proteins and are not present *in vivo* in a soluble form, except at their site of action (Schein, 1989; Sieker *et al.*, 1988; Racker, 1983). The dependence of inclusion body formation on the cell growth temperature has also been shown with heat-shocking cells by a few degrees above their normal growth temperature (Bishai *et al.*, 1987; Botterman and Zabeau, 1985; Haase-Pettingell and King, 1988; Nguyen *et al.*, 1989; Nover and Scharf, 1984; Piatak *et al.*, 1988; Gribskov and Burgess, 1983).

1.4.3.2 Properties and recovery of inclusion bodies.

Inclusion bodies are amorphous (Schoner *et al.*, 1985; Shoemaker *et al.*, 1985) or highly regular (Bowden *et al.*, 1991) protein aggregates, that are clearly separated from the rest of the cytoplasm but not surrounded by any membrane structure, as seen by transmission electron microscopy (Bowden *et al.*, 1991). They are dense, refractile, and visible under phase contrast-microscopy. They can be as large as a bacterial cell and change the single cell light scattering of producing organisms (Wittrup, 1988). Their size and density, probably determined by the polypeptide sequence and cellular factors, distinguishes them from other proteinaceous structures in prokaryotes (Taylor *et al.*, 1986).

The inclusion bodies consist primarily of the recombinant protein and non-reducible polymers thereof (Marston, 1986) and other cellular components. The material isolated as inclusion bodies often contains other cellular components. These may be an integral part of the inclusion bodies, be adsorbed on the surface of the protein particles following cell lysis, or simply co-precipitate with the inclusion bodies as an insoluble fraction (Bowden *et al.*, 1991). As a result, the four subunits of RNA polymerase, some combination of the outer membrane proteins OmpC, OmpF, and OmpA, 16S and 23S rRNA, and circular and nicked plasmid DNA are typical contaminants found associated with inclusion bodies (Hartley and Kane, 1988). The inclusion bodies may also contain the plasmid-encoded antibiotic resistance protein, as shown recently with the incorporation of pre-ß-lactamase during the overexpression of *Vitreoscilla* hemoglobin in *E. coli* (Rinas and Bailey, 1993).

Production of protein as an insoluble inclusion body offers the advantage of an easy purification. These particulate "organelles" may be isolated by a simple preliminary purification step such as differential centrifugation at low speed, thus bringing about a considerable purification of the product. A typical inclusion body recovery and purification scheme involves the removal of the outer membranes and the cell wall, prior to the preparation and differential centrifugation of the cell extract (Lin and Cheng, 1991). The treatment of the particles with deoxycholate and Triton X-100 has been shown to remove most of the contaminating material, but also some of the polypeptides that are an integral component of the aggregates, a problem that could be avoided by purification by a simple density gradient centrifugation (Bowden *et al.*, 1991).

1.4.4 Renaturation of polypeptide chains from inclusion bodies.

A common method used for recovery of native protein from insoluble inclusion bodies begins with the solubilization of aggregated polypeptide chains, followed by correct refolding of the polypeptides into the native conformation. The misfolded polypeptide chains in aggregates are stabilized largely by non-covalent intra- and intermolecular ionic and hydrophobic interactions, hydrogen bonds and sometimes by disulfide bonds (Marston, 1986; Schein, 1989). Therefore, aggregates are readily dissociated by exposure to strong denaturant such as guanidine hydrochloride or urea, high pH, neutral chaotropic salts (Lim *et al.*, 1989) and ionic detergents, with reduction of sulfhydryl groups if necessary.

The refolding of denatured polypeptide has been achieved by a variety of methods (Marston, 1986; Schein, 1989). The most common procedure involves the removal of the denaturant by dialysis (London *et al.*, 1974; Goldberg, 1985; Lim *et al.*, 1989). Other procedures that have been applied to renaturation of unfolded protein include the quick dilution method, resulting in the rapid reduction of the protein concentration and denaturant under refolding conditions (Rinas *et al.*, 1992), and the folding assisted by antibody (Carlson and Yarmush, 1992), ligand or co-solvent (Cleland and Wang, 1990). Renaturation has also been carried out using the bacterial molecular chaperone GroEL (Brown *et al.*, 1992) as the folding aid *in vitro*. Other procedures have involved the refolding of proteins reversibly bound to solid supports. For proteins where intermolecular interactions might be destructive as with proteinases, immobilization of the the proteins on a solid phase has been used for the refolding (Epstein and Anfinsen, 1962; Janolino *et al.*, 1978; Sinha and Light, 1975). The yields of native protein from all these procedures can range from very low to nearly quantitative (Nilsson and Anderson, 1990).

1.5 Research objectives and rationale.

The objectives of this project were to immobilize ACV-synthetase from *S. clavuligerus*, then to optimize and use the immobilized enzyme system for the *in vitro* synthesis of ACV, as a potential alternative to chemical synthesis. A cost analysis of this immobilized ACV-synthetase-based process was to be carried out to assess its economic feasibility. Since the optimization of the ACV production process requires a good understanding of the characteristics of the enzyme, another objective of this research project was to study the biochemical and stability properties of *S. clavuligerus* ACV-synthetase, as well as the kinetics of production of the enzyme.

At the time of starting this work, the following was known or assumed about the *S. clavuligerus* ACV-synthetase, as a peptide synthetase. First, the enzyme has a transient appearance during the growth of the culture (i.e. very short half-life) *in vivo*, thus there was need to establish the conditions for harvesting mycelia to maximize enzyme yields. Second, the enzyme was also very unstable *in vitro*. Therefore, there was need to define the conditions that maximize stability of the enzyme, by investigating enzymic chemical and physical parameters. The only reports on the partial stabilization of ACV-synthetase at that time were by Banko *et al.* (1986, 1987). Two possible strategies were: (i) immobilize the enzyme initially despite its lability *in vitro* with the intent that the immobilization would ultimately improve the stability of the enzyme; or (ii) carry out a systematic analysis of conditions that would improve the stability of the enzyme first, then immobilize the enzyme under the optimized conditions. The latter was adopted, for it had the advantage of enabling increase in the understanding of the enzyme and its producing organism through the initial physiological studies aiming at optimizing the enzyme yield.

Subsequent areas of studies involved two projects. One aimed at investigating the effect on ACV-synthetase of ACVSR, a 32-kDa protein that co-purified with ACV-synthetase (Jensen *et al.*, 1990), and that was believed to co-immobilize with the enzyme. The other research project pertained to the overexpression of the DNA fragment coding for the putative domain-1 of ACV-synthetase in *Escherichia coli* and the characterization of this polypeptide.

2. MATERIALS AND METHODS.

2.1 Bacterial strains and culture conditions.

Streptomyces clavuligerus NRRL 3585 from the Northern Regional Research Laboratories, Peoria, Illinois, was maintained as lyophilized spores or spore suspensions in 20% glycerol at -75°C. *Escherichia coli* pOW409/JM109 (Yanisch-Perron *et al.*, 1985), was maintained as a 20% glycerol stock at -20°C. *Escherichia coli* Ess, was generously provided by Dr. A. L. Demain, Massachusetts Institute of Technology, Cambridge, MA, U.S.A, and was stored at -20°C. *Staphylococcus aureus* N-2, was stored as a 20% glycerol stock at -75°C.

2.1.1 *Streptomyces clavuligerus* NRRL 3585.

For typical growth of the organism, seed culture was prepared from 0.5 mL of a glycerol spore stock in 25 mL of Trypticase Soy broth supplemented with 1% (w/v) soluble starch (TCS/S) medium incubated at 28°C for 48 h (Jensen *et al.*, 1986). Production flasks were inoculated with 2% (v/v) of *S. clavuligerus* seed culture into TCS/S and grown at 28°C, with shaking at 250 rpm.

The studies on the effects of amino acids on the production of clavulanic acid and cephamycin by cultures of *S. clavuligerus* were carried out in defined media supplemented with the amino acid (Romero *et al.*, 1984). Two millilitres of thawed mycelium suspension were used to inoculate 50 mL of seed medium consisting of malt extract (10 g/L), Bacto peptone (10 g/L), glycerol (20 g/L), pH 7 in 250-mL baffled flask, and incubated for 48 h at 28°C. Washed mycelia were then used to inoculate at 5% (v/v) the glycerol plus proline-based production medium containing: glycerol (15 g/L), sucrose (20 g/L), proline (2.5 g/L), NaCl (5 g/L), K_2HPO_4 (2 g/L), $CaCl_2$ (0.4 g/L), $MnCl_2.4H_2O$ (0.1 g/L), $FeCl_3.6H_2O$ (0.1 g/L), $ZnCl_2$ (0.05 g/L), $MgSO_4.7H_2O$ (1 g/L), and distilled water, pH 7.0. The glycerol plus proline-based medium (50 mL in 250-mL baffled flask or 100 mL in

500-mL flask) was supplemented with one of the following: arginine, glutamic acid or ornithine, at the final concentration of 1.5 g/L (Romero *et al.*, 1986). The mineral salts were sterilized separately as a 10x concentrated stock solution and then added to the medium prior to inoculation to avoid the precipitation of some medium components. The effects of the amino acids were also studied using the modified starch plus asparagine based medium of Aharonowitz and Demain (1977) which contained soluble starch (10 g/L), L-asparagine (2 g/L), 3-(N-Morpholino)propane-sulfonic acid (MOPS) buffer (21 g/L), $MgSO_4.7H_2O$ (0.6 g/L), K_2HPO_4 (4.4 g/L), trace mineral salts [1 mL of solution containing $FeSO_4.7H_2O$ (0.1 g), $MnCl_2.4H_2O$ (0.1 g), $ZnSO_4.H_2O$ (0.1 g), and $CaCl_2$ (0.1 g) per 100 mL of distilled water], and distilled water, pH 6.8. All cultures were grown at 28^oC, with shaking at 250 rpm.

2.1.2 *Escherichia coli* pOW409/JM109 expression system.

The DNA fragment encoding the putative first domain of *S. clavuligerus* ACV-synthetase was kindly provided by Dr. S. Kovacevic (Eli Lilly, Indianapolis, IN) on a 9.4 kb plasmid, pOW409, which contains the tetracycline resistance gene as a selection marker. This putative first domain of ACV-synthetase, on an approximately 3 kb *Kpn*I-*Bam*HI fragment, was cloned into the *Nde*I-*Bam*HI expression vector backbone from pOW382 (Kovacevic *et al.*, 1989). The NdeI site was introduced by linker mutagenesis to allow transfer of the gene to an *E. coli* high level expression system. The gene was placed under the control of the P_L promoter for heat induction, and the plasmid was introduced in *E. coli* strain JM109, to make the *E. coli* pOW409/JM109 expression system.

Initially, a seed culture of *E. coli* pOW409/JM109 was grown overnight at 28-30^oC with shaking in Luria Broth (LB) containing: tryptone (8 g/L), yeast extract (5 g/L), NaCl (5 g/L), and tetracycline (5 mg/L), supplemented with tetracycline (5 mg/L). The production culture (100 mL in 500 mL flask) was started with a 1% (v/v) inoculum of the overnight seed culture. The culture was allowed to grow with shaking at 250 rpm for 4 h at

28-30°C, then induced by increasing the growth temperature to 42°C for 2 h, before the cells were harvested. Other media were also investigated for the production of ACV-synthetase domain-1 polypeptide as described in the results.

2.2 Cell-free extract preparations.

S. clavuligerus cell-free extracts were routinely prepared for the isolation of ACV-synthetase. For time-course studies, 100-mL amounts of culture were harvested in duplicate at different times of growth. The mycelia were collected by filtration through Whatman No. 1 filter paper, and the filtrate saved for antibiotic assay. Mycelia from each flask were then washed with 50 mM Tris/HCl buffer, pH 7.5 containing 50 mM KCl; resuspended in 4 mL of MKG lysis buffer (100 mM MOPS-KOH buffer, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 20 mM ethylenediaminetetraacetic acid (EDTA), and 50% glycerol) supplemented with ß-mercaptoethanol (30 mM), and disrupted by sonication to make crude cell-free extracts (Jensen *et al.*, 1986). For proteinase analysis, $MgCl_2$ and EDTA were omitted from the lysis buffer. The cell-free extract was centrifuged for 30 min at 17,000 x g, and the clarified cell-free extract was then stored at -75°C until use. Time-course experiments were carried out three times.

E. coli pOW409/JM109 cell-free extracts were prepared for the isolation of ACV-synthetase domain-1 polypeptide inclusion bodies. Cells were recovered from 1 L of culture by centrifugation at 10,000 x g for 10 min at 4°C. The cells were resuspended in 0.5 L TDEG buffer [50 mM Tris/HCl, pH 8.0, 1 mM dithiothreitol (DTT), 0.01 mM EDTA and 10% glycerol], washed, centrifuged and resuspended in 20 mL TDEG buffer. The cells were split in 4x 5-mL portions that were alternately sonicated with two 10-sec pulses at power 7 with 1 min-cooling, to allow sufficient cooling times. The total sonication time for each fraction was 80 sec, using a Sonifier Cell Disruptor 350 (SmithKline Co., USA). The sonicated cells were centrifuged, pellet resuspended in 20 mL TDEG buffer, and the sonication and washing procedure repeated for a total of 3 sonications. The domain-1 inclusion bodies were recovered as a pellet after the third sonication, resuspended in 3 mL TDEG buffer, and small portions (0.1 - 0.2 mL) were stored at -75°C until use. Alternatively, inclusion bodies were isolated from sonicated sphaeroplasts, following a treatment of the inclusion body preparation with DNase, RNase and Triton X-100 to remove contaminating DNA, RNA and cell membrane-bound proteins (Lin and Chen, 1991).

2.3 Inclusion body solubilization and refolding of ACV-synthetase domain-1.

Inclusion body preparation (0.1 - 0.2 mL) was thawed and centrifuged in a microfuge (MSE, Johns Scientific Inc., Canada) at 13000 rpm, for 10 min. The pellet was washed twice with 1 mL of MOPS/KOH buffer (100 mM, pH 7.5) containing 2 mM DTT, followed by centrifugation. The aggregates (3 mg protein) were solubilized in a denaturation buffer [100 mM MKG buffer, pH 7.5, DTT (50 mM), EDTA (5 mM) and urea (5-8 M)], for 2 h at room temperature. The solubilized protein was centrifuged, and the clear supernatant was loaded, desalted and renatured on a 10-mL desalting column (Econo-Pac 10DG, Bio-Rad, Richmond, CA). Small amounts of this desalted solution of domain-1 polypeptide were analyzed for activity using the ^{14}C-amino acid binding assay. Optimized conditions are described in the results.

2.4 Enzyme purification.

ACV-synthetase was purified by a combination of salt precipitation, ultrafiltration, and anion-exchange chromatography as described by Jensen *et al.* (1990). Partial purification was achieved by treatment of the cell-free extracts, diluted four-fold in MDK buffer [100 mM MOPS-KOH, pH 7.5, 1 mM DTT, 50 mM KCl], with streptomycin sulfate (1% w/v), followed by ammonium sulfate fractionation. ACV-synthetase was obtained in the 35-45% saturation ammonium sulfate precipitate, and resuspended in MDKG buffer [MDK buffer containing 20% glycerol (v/v)].

Purification of ACV-synthetase from ACVSR, a 32-kDa protein that co-purified with ACV-synthetase (Jensen *et al.*, 1990), was achieved by gel filtration chromatography on a Superose 6B HR 16/50 prepacked column (Pharmacia, Baie d'Urfe, PQ), with some modifications of the procedure described by Aidoo *et al.*(manuscript in preparation). The fractions from the anion-exchange column (Mono Q HR 5/5, Pharmacia, Upsalla) containing ACVSR and ACV-synthetase were pooled (3.0 mL) and concentrated 6-fold by ultrafiltration in a Centricon XM 30 (Amicon, Beverly, MA) at low speed. The protein solution was then loaded on the Superose 6B column attached to a fast liquid chromatography system (FPLC, Pharmacia, Baie d'Urfe, PQ) and equilibrated with MD buffer containing 10% glycerol. Elution was carried out with the same buffer at 0.3 mL/min.

2.5 Enzyme assays.

2.5.1 ACV-synthetase.

ACV-synthetase activity was measured in standard reaction mixtures (0.1 mL) consisting of ATP (2 mM), $MgCl_2$ (6 mM), DTT (5 mM), phosphoenolpyruvate (PEP, 5 mM), L-α-aminoadipic acid (5 mM), L-cysteine (1 mM) and L-valine (5 mM), in 100 mM Tris/HCl, pH 8.5 buffer. This enzyme assay was modified from that initially described by Jensen *et al.* (1988) and which contained ATP at 1 mM, $MgCl_2$ at 3 mM, DTT at 2 mM and pyruvate kinase at 6.5 units in 100 mM MOPS/KOH, pH 7.5 buffer (Table 2.1). In studies optimizing the ACV-synthetase assay, this initial assay was used as the basic condition, with Tris/HCl pH 8.5 instead of MOPS/KOH, pH 7.5 serving as the buffer. Assays were incubated for 1 h at 22-24°C, terminated with 0.025 mL of 20% trichloroacetic acid (TCA), and precipitated protein was removed by centrifugation. ACV in the mixtures was derivatized with Thiolyte MB, a fluorescent thiol reagent, and quantitated by HPLC as

Table 2.1. Initial ACV-synthetase assay mixture.

Component (1)	Concentration (1)	Component (1)	Concentration (1)
ATP	1 mM	L-α-aminoadipate	5 mM
$MgCl_2$	3 mM	L-cysteine	1 mM
DTT	2 mM	L-valine	5 mM
PEP	5 mM	MOPS/NaOH, pH 7.5	100 mM
		Pyruvate kinase	6.4 units/mL

(1) The components and their concentrations as described in the initial assay (Jensen *et al.* 1988). The mixtures (0.1 mL) including the enzyme were incubated for 1 h at room temperature, and ACV formed analyzed by HPLC.

described below. One unit of ACV-synthetase activity was defined as the amount producing 1 µmol of ACV per min, and specific activity was expressed as munit/mg protein.

Enzyme activity in purification fractions was determined with a 5-fold scaled down assay system consisting of 10 µL enzyme and 10 µL of a mixture of concentrated reagents. The reaction was for 30 min at room temperature, and the activity under these conditions was expressed as a percent relative to the peak fraction activity.

The ACV content of reaction mixtures was quantitated by HPLC as described previously (Jensen *et al.*, 1988), with slight modifications. The following equipment was used: M-45 and M-6000A solvent delivery system, WISP Model 710 automatic injector, Model 441 fluorescence detector, Model 840 system controller and a µBondapak column in a Z module, all from Waters Scientific Co., Mississauga, Ont., Canada. The solvent system consisted of solvent A [10% methanol : 0.025% (v/v) acetic acid adjusted to pH 5.0 with NaOH] and solvent B [90% methanol : 0.025% (v/v) acetic acid adjusted to pH 5.0 with NaOH]. Ten-microliter amounts of Thiolyte-derivatized samples were analyzed at a flow rate of 1.5 mL/min, and the eluted components detected by fluorescence using a 360 nm excitation filter and a 455 nm emission filter. The elution scheme of a typical 30-min run consisted of a 15-min linear gradient varying from 92% A : 8% B to 60% A : 40% B, followed by a 10-min isocratic elution at 60% A : 40% B, and a brief increase of B to 100% before resuming initial conditions. Under these conditions, authentic ACV eluted with a retention time of 15.2 min, and the estimation of ACV concentrations in reaction mixtures was based on comparison of peak areas with those of ACV standards.

2.5.2 ACV-synthetase domain-1 binding.

ACV-synthetase domain-1 activity was determined by the binding of ^{14}C-amino acid. Reaction mixtures (0.2 mL) contained: ATP (5 mM), $MgCl_2$ (6 mM), DTT (3 mM), 0.125 µCi of DL-(6-^{14}C)-α-aminoadipic acid (55 mCi/mmol), L-(U-^{14}C)cysteine (153.1

mCi/mmol), or L-(U-^{14}C)valine (288.5 mCi/mmol), in 100 mM Tris/HCl, pH 8.5 buffer. The reaction was allowed to proceed at room temperature for 30 min, before stopping it with 0.2 mL TCA (10%) and protein recovered by centrifugation. The precipitated protein was washed three times with 0.4 mL TCA (5%), and once with ethanol, with vigorous mixing and centrifugation between each wash. The protein was then redissolved in 0.1 mL formic acid, diluted two-fold with water, before the addition of the fluor mixture (Aqueous Counting Scintillant, Amersham, Oakville, ON) for measurement of radioactivity in a Beckman Liquid Scintillation Counter (Beckman Instruments Inc., Fullerton, CA).

2.5.3 Isopenicillin N synthase.

The conversion of ACV to isopenicillin N was measured as described before (Jensen *et al.*, 1986). The reaction mixtures contained *bis*-ACV (0.287 mM, prereduced with DTT), DTT (4 mM), sodium ascorbate (2.8 mM), $FeSO_4$ (45 µM) , Tris/HCl buffer (0.05 mM, pH 7.5), and enzyme to give a final volume of 0.04 mL. The reaction mixtures were incubated for 1 h, at room temperature and terminated by the addition of 0.04 mL of methanol. Isopenicillin N formation was estimated from the amounts of ACV removed, quantitated by HPLC (Jensen *et al.*, 1982b). One unit of IPNS activity was defined as that amount of enzyme which produces 1 µmol of isopenicillin N per min.

The isocratic elution of ACV from the IPNS reaction mixtures, was achieved with an M 6000A pump and detected with a M-490 variable wavelength detector. The mobile phase consisted of methanol-potassium phosphate buffer (pH 4.0) 0.05 M (5 : 95). Twenty-microliter amounts of reaction mixtures were analyzed at a flow rate of 2 mL/min. Detection was carried out at both 220 nm and 260 nm with a sensitivity setting of 0.02 absorbance units full scale (Jensen *et al.*, 1982b).

2.5.4 Proteinase.

The proteinolytic activity of *S. clavuligerus* crude cell-free extracts was measured using a modified assay based on the proteinolytic digestion of azocasein (Rollins *et al.*, 1988). The cell-free extract was preincubated at 35°C for 2 min, and 0.1 mL of the extract was added to a reaction mixture containing 0.3 mL 100 mM Tris/HCl buffer, and 0.3 mL 2% azocasein (Sigma, St Louis, MO, USA), at 35°C. The reaction was allowed to proceed for 1 h, and stopped with 0.7 mL 15% TCA. The colored TCA-soluble material was quantitated spectrophotometrically. One unit of proteinase activity was defined as the amount producing 1 absorbance unit per min at 335 nm, and specific activity was expressed as munit/mg protein.

Proteinase inhibition was studied using: phenanthroline, phenylmethylsulfonyl fluoride, and thioacetamide as concentrated solutions in ethanol; and EDTA and *p*-chloromercuriphenyl-sulfonic acid were dissolved in 100 mM Tris/HCl pH 8.2. Inhibitor solutions were added to the cell-free extracts, pre-incubated for 10 min at 35°C, and residual proteinolytic activity was then determined with azocasein for 1 h at 35°C.

2.6 Protein analysis.

Protein was assayed according to the protein dye-binding method of Bradford (Bradford, 1976) (BioRad, Richmond, CA, USA), or the Lowry procedure for the estimation of proteins (Lowry *et al.*, 1951) using bovine serum albumin as a standard.

2.7 Polyacrylamide gel electrophoresis.

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the procedure of Laemmli (1970), and gels were stained with Coomassie Brilliant blue R-250. Non-denaturing gels were carried in the absence of SDS and ß-mercaptoethanol.

2.8 Bioassays of ß-lactam antibiotics.

The total antibiotic (cephamycin and cephalosporin) content in the culture fluid was determined by the agar diffusion method using the super-sensitive *Escherichia coli* strain Ess, and the concentration estimated from cephalosporin C standards (Jensen *et al.*, 1982b).

The ß-lactamase inhibitory activity of clavulanic acid was assayed in plates (20 x 20 cm) using 100 mL TCS agar containing penicillin G (1 µg/mL) and seeded with 100 µL of a 20%-glycerol stock of the penicillinase producing *Staphylococcus aureus* N-2, with an OD_{600} of 7.0 (Jensen *et al.*, 1982b). Ten to twenty microliters of centrifuged *S. clavuligerus* culture fluids were spotted on sterile 6.5 mm filter paper discs (Schleicher and Schuell, NH, USA) placed on the agar, and the zones of inhibition were measured after incubation of the plates for 24 h at 37°C. The concentration of clavulanic acid in culture fluid was estimated from clavulanic acid standards.

2.9 Thin layer chromatography.

Products from ATP utilization by ACV-synthetase in reaction mixtures was determined with ^{32}P-ATP (α- or γ-labelled, ICN Biomedicals, Inc., Irvine, CA). Reaction mixtures (0.03 mL unless stated otherwise) contained: ATP (0.5 mM), $MgCl_2$ (6 mM), DTT (5 mM), PEP (5 mM), L-α-aminoadipic acid (5 mM), cysteine (1 mM) valine (5 mM), and 0.25-0.50 µL of γ-^{32}P-ATP (4500 Ci/mmol, 10 mCi/mL) or α-^{32}P-ATP (3000 Ci/mmol, 10 mCi/mL) and Mono Q purified ACV-synthetase (20 µL), in 100 mM Tris/HCl, pH 8.5 buffer. When AMP, Ca^{+2} and PEP were supplemented to the reaction mixture, they were used at 5 mM, 3 mM and 5 mM, respectively. The reaction mixtures were incubated at room temperature for 30 min before the analysis of products. Negative controls consisted of enzyme heated for 15 min, at 95-100°C. Reaction mixtures were stopped by immediately spotting 3 µL aliquots on a polyethyleneimine (PEI) cellulose sheet, prepared on plastic sheets according to a procedure described by Randerath and

Randerath (1967). The nucleotides and inorganic phosphates were resolved by thin layer chromatography (TLC), using a solvent system consisting of LiCl (0.33 M), formic acid (0.67 M), and EDTA (1.67 M). After 45 min of development, the chromatogram was air-dried and nucleotide spots visualized by autoradiography using a Kodak Scientific Imaging film XAR. Unlabelled nucleotide spots were visualized under U.V. light at 254 nm.

2.10 Derivatization of controlled pore glass.

Controlled-pore glass (CPG-240, 240-Å pore size and 200-400 mesh size) was aminated as described before (Kadima and Pickard, 1990). Glass beads (1 g) were first acid washed by heating for 1 h in 10% nitric acid at 80-90°C, extensively washed with water, and then heated in 20 mL of 10% aqueous aminopropyltriethoxysilane solution, pH 3.4 (Weetall, 1976), for 3 h at 70°C. The aminopropyl-glass was washed with water on a sintered glass filter, air dried, then dried overnight in oven at 95-100°C. The aminopropyl-glass was again washed with water before crosslinking with glutaraldehyde or used as such as an ionic binding matrix (Kadima, 1989).

2.11 Immobilization of ACV-synthetase

Purified ACV-synthetase was immobilized by ionic bonding onto DEAE-Trisacryl resin (Jensen *et al.*, 1989), and on aminopropyl glass (Kadima and Pickard, 1990).

ACV-synthetase was also immobilized by physical confinement in a porous ultrafiltration reactor (50 mL Amicon containing a PM-10 or YM-30 membrane), using the partially purified enzyme (Jensen *et al.*, 1990). The enzyme was added to a scaled-up ACV-synthetase assay mixture (5 mL) and incubated with gentle mixing at 24°C, under either an oxygen or nitrogen atmosphere at an operating pressure of 15 psi. Samples (0.5 mL) were collected from the outlet tubes from which 100-µL volumes in duplicate were removed for analysis, and the unused portion was returned to the reactor. In experiments testing the reusability of the immobilized enzyme, the reaction mixture was removed by ultrafiltration under pressure, and the enzyme (in 1 mL) washed twice with 5 mL of 20 mM Tris/HCl buffer, pH 8.5 containing 2 mM DTT and 10% glycerol before replenishment of the reactor with fresh reagents.

2.12 Radioisotopes, chemicals and matrices.

DL-(6-^{14}C)-α-aminoadipic acid from Cen Saclay (France). L-(U-^{14}C)cystine , and L-(U-^{14}C)valine were from Dupont (Mississauga, ON). Monobromobimane (Thiolyte MB) was from Calbiochem (San Diego, CA, USA). ACV was from Incell Corporation (Millwaukee, WI, USA). Penicillinase (Bacto-penase concentrate, 107 units/mL) was from Difco Laboratories, Detroit, MI. Penicillin G (potassium salt) and cephalosporin C (sodium salt) were gifts from D. Hook, Bristol Laboratories, Syracuse. Clavulanic acid was a gift from A. Brown, Beecham Pharmaceuticals, Betchworth, Surrey, U.K. Triton X-100 was from Rohm & Haas, West Hill, ON. Tween 80 was from Fisher Scientific, Fair Lawn, NJ. L-α-aminoadipic acid, ATP (disodium salt), ascorbic acid (sodium salt), DTT, pyruvate kinase and CPG-240 (240-Å pore size and 200-400 mesh size) were from Sigma Chemical Co., St Louis, MO. The resins DEAE-Trisacryl and CM-Trisacryl were from LKB Instruments, Inc., Rockville, MD.; DEAE-Sephacel was from Pharmacia Fine Chemicals Inc., Piscataway, NJ.; and CM-52 cellulose was from Whatman Biosystems Ltd., Fairfield, NJ. All other chemicals were of reagent grade.

3. RESULTS.

3.1 Production kinetics and stability properties of ACV-synthetase from *Streptomyces clavuligerus.*

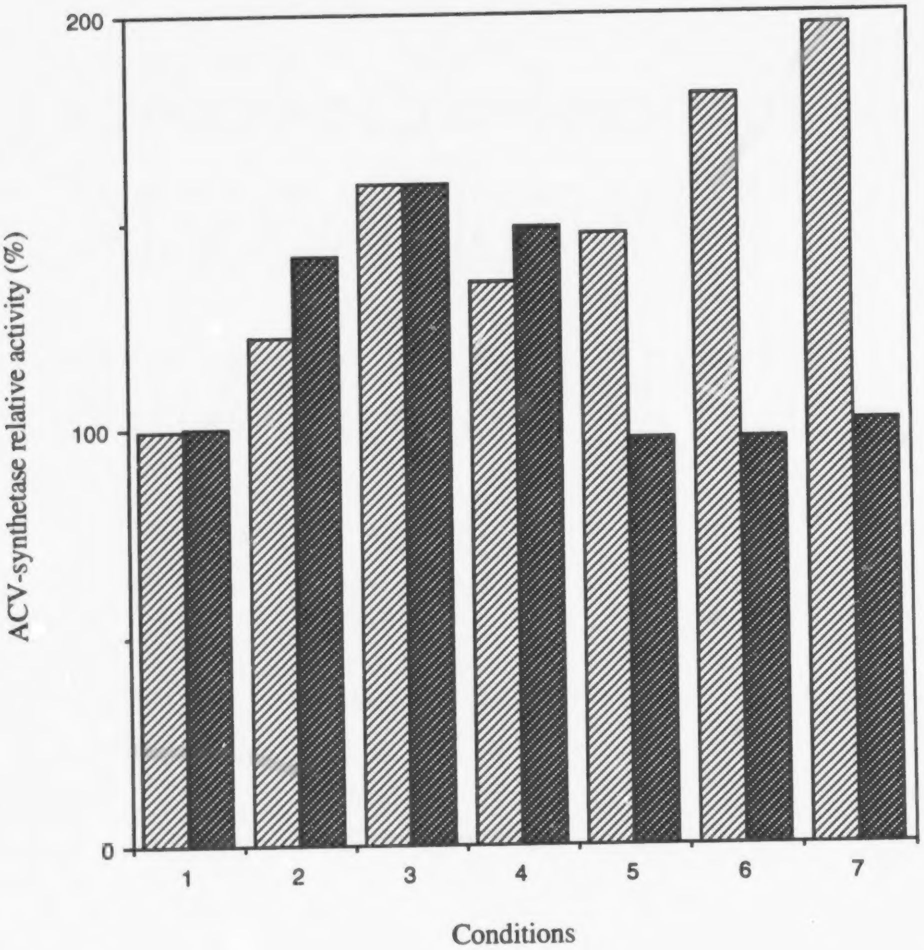
At the time of the beginning of these studies, little was known about the stability properties of ACV-synthetase from *S. clavuligerus*. The only reports on the stability of ACV-synthetase was by Banko *et al.* (1986, 1987), in which the enzyme from *Cephalosporium acremonium* was stabilized with glycerol. Although DTT was used in the preparation of active extracts in this laboratory, no systematic study had been carried out. A standard laboratory protocol was developed in which the enzyme was isolated from 44 h-old cultures and stabilized in a MOPS buffer containing glycerol and DTT or ß-mercaptoethanol. The enzyme was handled at 4°C and stored at -75°C. These practices retained sufficient activity for short term experiments, but were considered likely to be unsuitable for processes requiring highly stable enzyme with optimal specific activity to be used in the production of ACV. A systematic analysis of the culture harvest time and stabilization conditions was therefore necessary to optimize both yield and stability of the enzyme. However, the initial studies looked at the enzyme reaction conditions, which had been developed with crude ACV-synthetase, to confirm that they were also optimal for purified enzyme. Parts of these studies have been published (Kadima *et al.*, 1993).

3.1.1 Dependence of the phosphoenolpyruvate (PEP) effect on the purity of ACV-synthetase.

A study analyzing the effect of ATP, PEP and other assay components on the activity of ACV-synthetase was carried out with both partially purified and purified enzymes. Increasing the ATP concentration from 1 to 5 mM resulted in an increase of the detectable activity, but higher ATP concentrations were found to be inhibitory to both partially purified and purified enzyme (Fig. 3.1). However, when increasing amounts of

Figure 3.1 Analysis of the PEP-mediated stimulation of ACV-synthetase activity: effects of ATP and PEP.

Partially purified (0.64 mg, 0.16 mU/mg, obtained after an ammonium sulfate fractionation) (▨) and purified (0.12 mg, 0.77 mU/mg, obtained after a second Mono Q column) ACV-synthetase (■) were used to analyze the effects of ATP and PEP on ACV-synthetase activity. The reaction mixtures were as described by Jensen *et al.* (1988) but without pyruvate kinase, and at ATP concentrations of 1 mM (**1**), 2 mM (**2**), 5 mM (**3**), and 10 mM (**4**) in the absence of PEP; and at PEP concentrations of 1 mM (**5**), 5 mM (**6**) and 10 mM (**7**) at a constant ATP concentration of 1 mM. The activity of the enzyme in condition **1** for partially and purified enzymes was set at 100%. The experiment was carried out three times in duplicate.


ACV-synthetase relative activity (%)
200
100
0
1
2
3
4
5
6
7
Conditions

PEP were supplemented to the 1 mM-ATP assay system, further stimulation of activity of the partially purified enzyme was observed, and to levels that were higher than with elevated concentrations of ATP alone. No similar effect was observed with purified enzyme. In addition, no significant stimulatory effect by pyruvate kinase (6.5 units) was detected with either partially or highly purified enzyme, suggesting that the stimulatory effect observed was likely mediated by factors present in the cell-free extracts, and not by the added pyruvate kinase.

As a result, enzyme preparations of different purity were analyzed to determine the extent of the PEP-mediated stimulation of ACV-synthetase activity. It was found that this effect decreased with increasing purity of ACV-synthetase. The strongest stimulatory effect by PEP was observed with crude cell-free extract, whereas the highly purified enzyme, showed no effect at all (Table 3.1). In an attempt to characterize the nature of the PEP stimulatory effect, an endogenous PEP-dependent ATP regenerating system, such as the pyruvate kinase plus adenylate kinase coupling system was assumed to be involved. To test this possibility, the effects of Ca^{+2}, a strong inhibitor of pyruvate kinase (Betts *et al.*, 1968; Mildvan and Cohn, 1967), and the product pyruvate on the activity of partially purified and purified ACV-synthetase were analyzed. As shown in Figure 3.2, Ca^{+2} was more inhibitory to the partially purified enzyme than to the purified enzyme, whereas high concentrations of pyruvate – presumed to exert a strong product inhibition – had no effect on the enzymes. The inhibitory effect of Ca^{+2} was also observed in the absence of PEP. Purified enzyme was affected by Ca^{+2} only slightly. As a result, the observed effect was believed to be partly due to a competition between Ca^{+2} and Mg^{+2} for ATP, even though Mg^{+2} was always used in excess or at equimolar amounts during these analyses. Because of the complexity of the partially purified enzyme system, no further conclusion could be drawn from these studies.

Further characterization of the enzyme assay indicated that a system consisting of 2 to 5 mM ATP and 5 mM PEP was optimal for estimating the activity of not only the crude

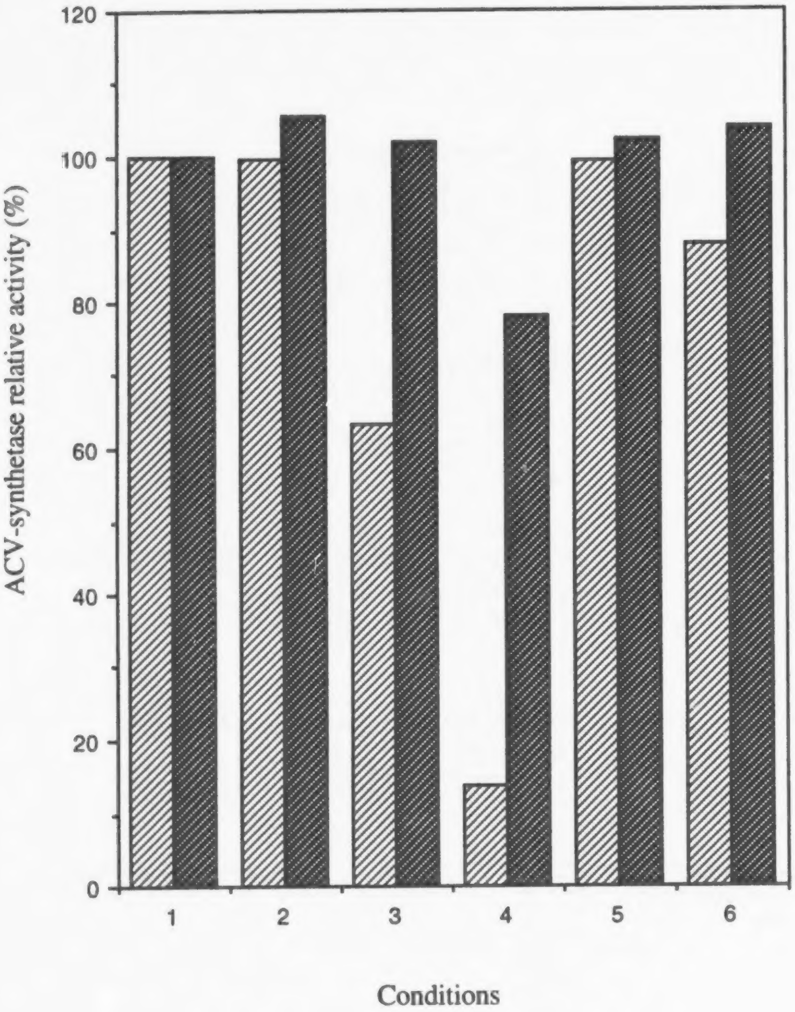
Table 3.1. Dependence of the PEP effect on the purity of ACV-synthetase.

	Relative ACV-synthetase activity (%)		
Purification step [1]	2 mM ATP	10 mM ATP	2 mM ATP/5 mM PEP
Crude cell extract	17	60	100
Ammonium sulfate (35 -45%)	45	75	100
Ultrafiltration (XM 300)	53	77	100
Purified (Mono Q, 2nd run)	103	109	100

(1) The activity of ACV-synthetase of different purity levels, obtained following the purification scheme previously described by Jensen *et al.* (1990), was measured in three assay systems and expressed as a % relative to the activity obtained with the assay system containing both ATP (2 mM) and PEP (5 mM).

Figure 3.2 Analysis of the PEP-mediated stimulation of ACV-synthetase activity: effects of Ca^{+2} and pyruvate.

Partially purified (0.64 mg, 0.16 mU/mg, obtained after an ammonium sulfate fractionation) (▨) and purified (0.12 mg, 0.77 mU/mg, obtained after a second Mono Q column) ACV-synthetase (■) were used to analyze the effects of Ca^{+2} and pyruvate on ACV-synthetase activity. The reaction mixtures were as described by Jensen *et al.* (1988) and supplemented with Ca^{+2} at the concentrations of 0 mM (**1**), 0.5 mM (**2**), 1 mM (**3**), and 5 mM (**4**); and pyruvate at the concentrations of 1 mM (**5**) and 10 mM (**6**). The activity of the enzyme in condition **1** for partially and purified enzymes was set at 100%. The experiment was carried out three times in duplicate.

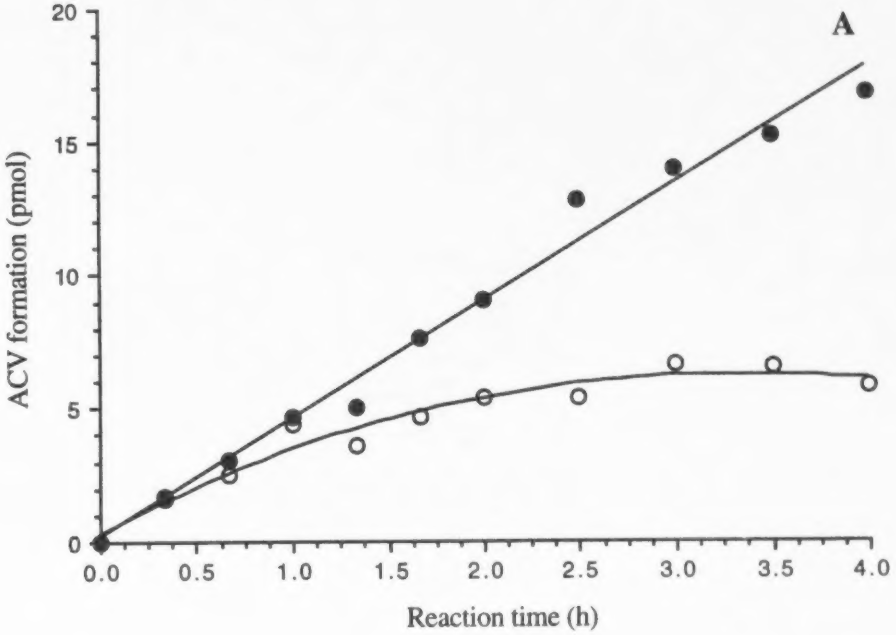

120
100
80
60
40
20
0
ACV-synthetase relative activity (%)
1
2
3
4
5
6
Conditions

but also the partially purified enzyme, and did not inhibit the highly purified enzyme. The importance of PEP as a component of the ACV-synthetase assay system was demonstrated during the kinetic analysis of ACV formation with partially purified enzyme in the presence and absence of of this reagent. The results indicated that the assay system containing PEP was capable of sustaining a linear reaction rate of ACV synthesis over a longer period of time (Fig. 3.3A) and with broader range of enzyme amount (Fig. 3.3B) than in the absence of PEP. These observations were useful for the development of an immobilized enzyme reactor for the production of ACV, using a partially purified ACV-synthetase.

3.1.2 Dynamics of cephamycin C fermentation.

To determine the growth period for harvesting mycelia to prepare cell-free extracts with optimum enzyme yields, a time-course study was carried out. ACV-synthetase activity was measured in crude cell-free extracts of *S. clavuligerus* growing in TCS/S medium, at 28°C. Since an established seed culture was used as inoculum, the onset of the culture growth was almost immediate, reaching stationary phase around 40 h (Fig. 3.4). The growth profiles, as measured by optical density at 600 nm and protein content of cell-free extracts, were very similar (Fig. 3.4A), indicating that either method was suitable for estimating growth of *S. clavuligerus*. ACV-synthetase activity and intracellular ACV were observed 8 h into the fermentation, and both increased up until 38-40 h (Fig. 3.4B). Intracellular ACV levels dropped, coinciding with the appearance of total antibiotic in culture fluids. Total antibiotic (cephamycin plus cephalosporin) production reached a maximum around 60 h and remained thereafter. However, ACV-synthetase activity remained high up to 96 h of fermentation, and analysis of the cell-free extracts by SDS-PAGE also showed the presence of a protein of the size of ACV-synthetase in younger as well as in older cultures.

Figure 3.3 Effect of PEP on the activity of partially purified ACV-synthetase. Effects of reaction time (**A**) and enzyme amounts (**B**) on ACV formation by partially purified ACV-synthetase (0.64 mg, 0.16 mU/mg) were analyzed with the standard assay system (●) or with the standard assay system without PEP (○).


A
ACV formation (pmol)
0
5
10
15
20
Reaction time (h)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0

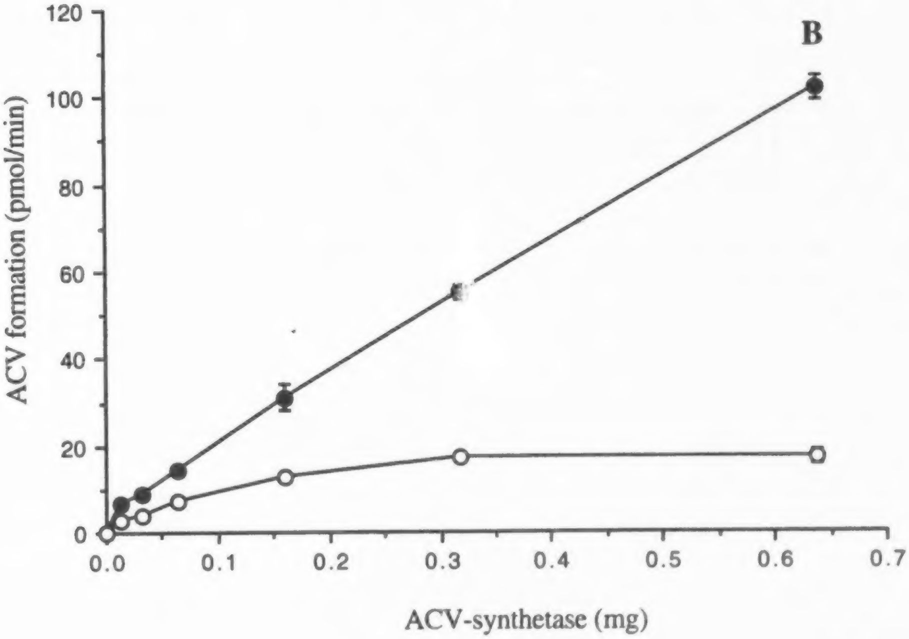
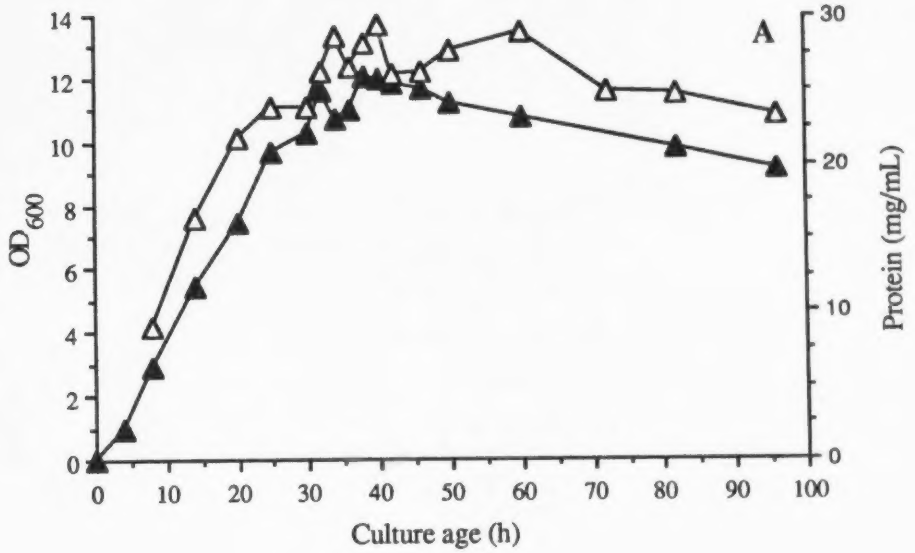
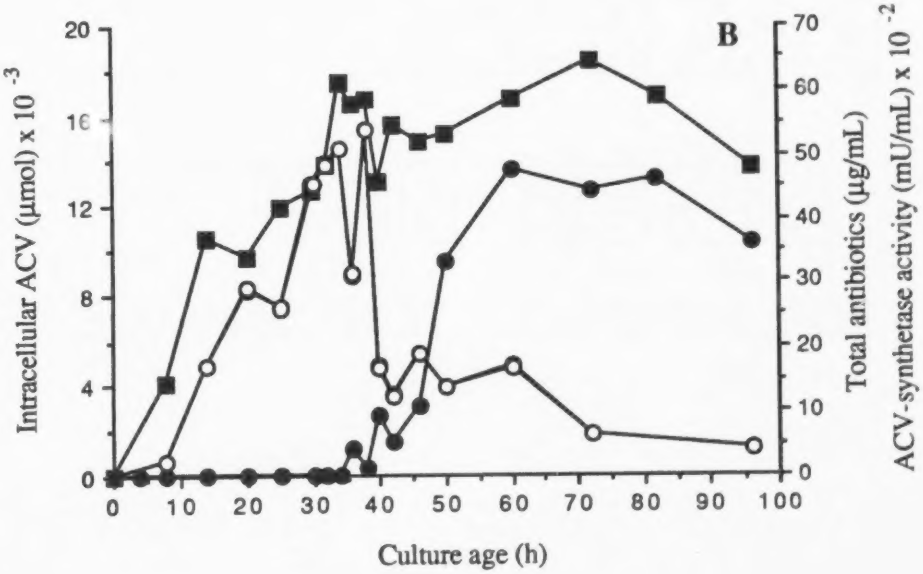

B
ACV formation (pmol/min)
0
20
40
60
80
100
120
ACV-synthetase (mg)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7

Figure 3.4 Dynamics of cephamycin C fermentation by *S. clavuligerus*.

A: Growth profile determined by OD_{600} of the culture (▲) and protein of crude cell-free extract (Δ). **B**: Time-course profiles of ACV-synthetase activity determined with the standard assay system (■), ACV (○) and total antibiotics (●) production.


A
OD$_{600}$
Protein (mg/mL)
Culture age (h)
0
2
4
6
8
10
12
14
0
10
20
30
0
10
20
30
40
50
60
70
80
90
100


B
Intracellular ACV (μmol) x 10 -3
Total antibiotics (μg/mL)
ACV-synthetase activity (mU/mL) x 10 -2
Culture age (h)
0
4
8
12
16
20
0
10
20
30
40
50
60
70
0
10
20
30
40
50
60
70
80
90
100

3.1.3 Effect of PEP on the detection of ACV-synthetase in older cultures.

The appearance of ACV-synthetase during the growth of *S. clavuligerus* was not transient: the enzyme was present during both trophophase and idiophase. This was unexpected and was in contrast with published activity profiles of ACV-synthetase from *S. clavuligerus* (Zhang *et al.*, 1989a,b). To determine whether the variation in time-course profiles was due to differences in the assay systems, the enzyme activities in crude cell-free extracts of different culture ages were determined in reaction mixtures containing ATP at 2 mM, 10 mM, and ATP at 2 mM plus PEP at 5 mM (Fig. 3.5). The results showed significant increases in the activity of ACV-synthetase with 2 mM ATP plus 5 mM PEP when compared with ATP alone at 2 or 10 mM. Using these reaction mixtures, variations in enzyme activity with culture age were also observed, resulting in the improvement of the detection of ACV-synthetase activity in older cultures. This is in contrast to an apparent decay in enzyme activity in older cultures when assayed without PEP (Fig. 3.5).

3.1.4 Dependence of the storage stability on the culture age.

The inactivation kinetics of gramicidin S synthetase from *B. brevis* have been shown to be strongly dependent on the growth stage of the culture (Tasker and Agathos, 1989). To analyze for any similar correlation between the physiology of the culture from which ACV-synthetase is obtained and its *in vitro* stability, crude cell-free extracts were prepared from cultures grown for different times. ACV-synthetase activity of these freshly prepared cell-free extracts was analyzed to determine initial activity (Fig. 3.6). The same samples were stored at 4°C and activity analyzed again after 1, 2 and 4 days to obtain stability profiles of the enzyme. The results indicated that the enzyme from mycelia harvested before 36-38 h of fermentation was most stable and retained most of its initial activity after 2 days of storage. A significant drop in residual activity after 4 days of storage was observed for cell-free extracts from cultures up to 38 h of growth, and no activity remained in cell-free extracts from 8-h cultures and older cultures.

Figure 3.5 Improvement of estimation of ACV-synthetase activity in crude extracts by PEP.

ACV synthetase activity in cell-free extracts prepared from cultures harvested at different times of the growth was analyzed with the standard assay system (○), standard assay system without PEP (●), and standard assay system without PEP and ATP at the concentration of 10 mM (□).

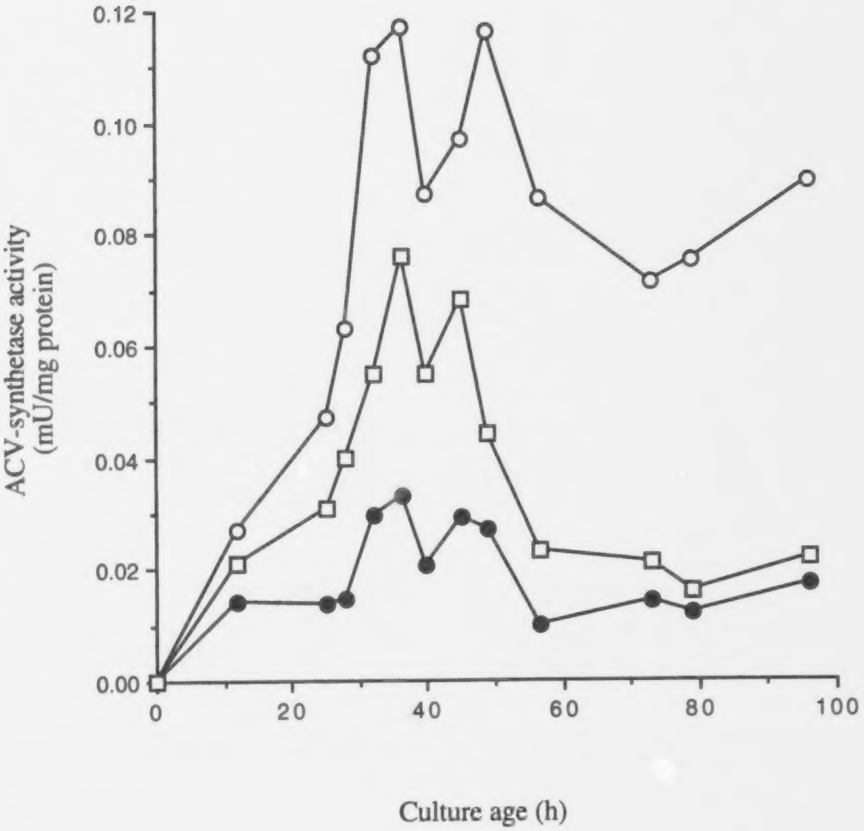
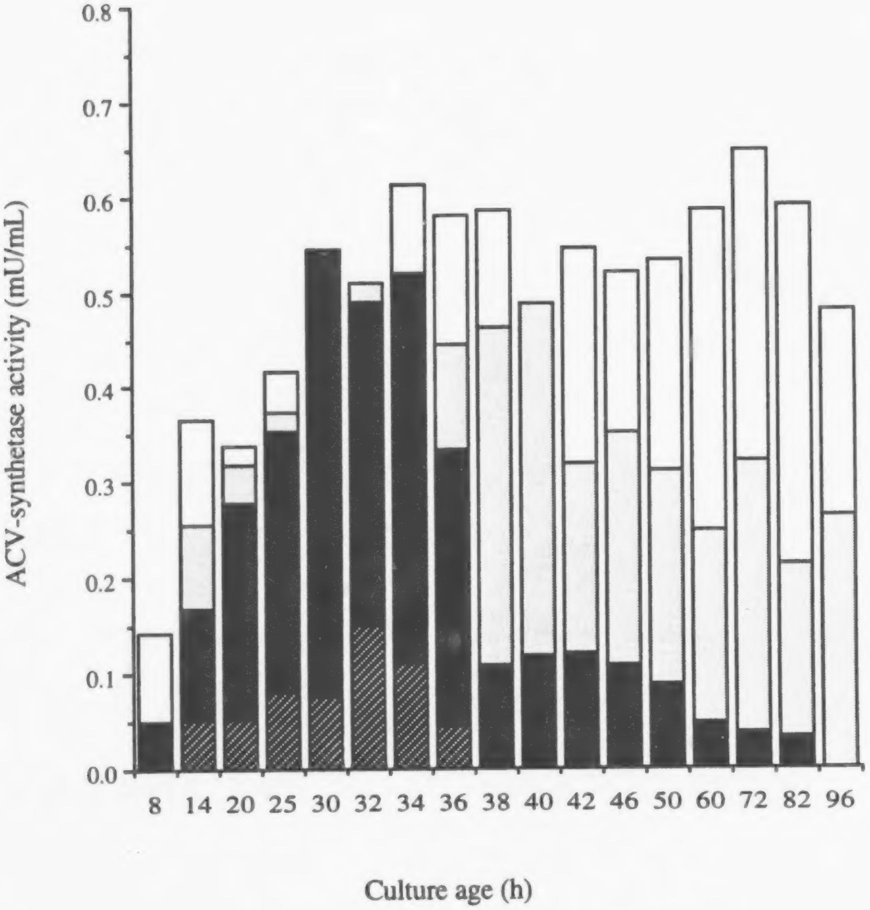

0.12
0.10
0.08
0.06
0.04
0.02
0.00
ACV-synthetase activity
(mU/mg protein)
0
20
40
60
80
100
Culture age (h)

Figure 3.6 Dependence of storage stability of ACV-synthetase on culture age. Initial ACV-synthetase activity of crude cell-free extracts from different age cultures was measured (□) before storage of the cell-free extracts at 4°C, then residual ACV-synthetase activity was measured after 1 (□), 2 (■) and 4 (■) days of storage.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ACV-synthetase activity (mU/mL)
8
14
20
25
30
32
34
36
38
40
42
46
50
60
72
82
96
Culture age (h)

3.1.5 Proteinolytic activity profile during cephamycin C fermentation.

Instability of peptide synthetases has been suggested to reflect intracellular proteinases (Kleinkauf and von Döhren, 1983), so crude cell-free extracts were also examined for proteinolytic activity on azocasein. A proteinase with an optimum activity at pH 8.2 was detected (Fig. 3.7). This proteinolytic activity increased with biomass, but no drastic change in specific activity was observed in the idiophase that correlated with the observed instability of ACV-synthetase in older cultures. Since this proteinase pH activity profile was determined with a 44 h culture cell-free extract and detected an alkaline proteinase, there was a possibility that an acidic or neutral proteinase also existed at a different stage of growth. The time-course samples were therefore assayed for azocaseinolytic activity at pH 5.7, but little in activity profile was observed, indicating that there was only one azocaseinolytic activity throughout the growth cycle. To see whether the proteinase activity of cell-free extracts changed during storage, the crude cell-free extracts from cultures grown for various times were incubated for 3 days at 4°C and the proteinolytic activity profile examined again. A nearly two-fold increase in proteinolytic activity was observed (Fig. 3.8), presumably as a result of activation of pre-proteinases during the storage period. Again, this effect was not localized to a particular growth period.

To determine the nature of the proteinase, a variety of specific inhibitors were used. At concentrations of up to 2 mM, no strong inhibitory effect was observed with any of the serine-, metallo- and thiol-proteinase inhibitors tested. Since proteinase activity may affect ACV-synthetase activity, after the failure to inhibit this activity attempts were made to separate these activities during the normal ACV-synthetase purification. Analysis of azocaseinolytic activity of samples from the ammonium sulfate precipitation procedure showed that most of the detected proteinase activity (95%) was in the supernatant and separated from ACV-synthetase activity, most of which precipitated in the 35-45% saturation range of ammonium sulfate. Negligible proteinolytic activity (1%) remained associated with the partially-purified ACV-synthetase. Despite the removal of most of the

Figure 3.7 pH activity profile of *S. clavuligerus* crude cell-free extract proteinase. The pH activity profile of proteinase in the cell-free extracts was assayed as described in section 2.5.4, with the following buffer systems: 100 mM glycine/HCl (pH 3.0-5.0), 100 mM KH_2PO_4/K_2HPO_4 (pH 5.0-7.5), 100 mM Tris/HCl (pH 7.5-8.5), and 100 mM triethanolamine (pH 8.5-9.5).

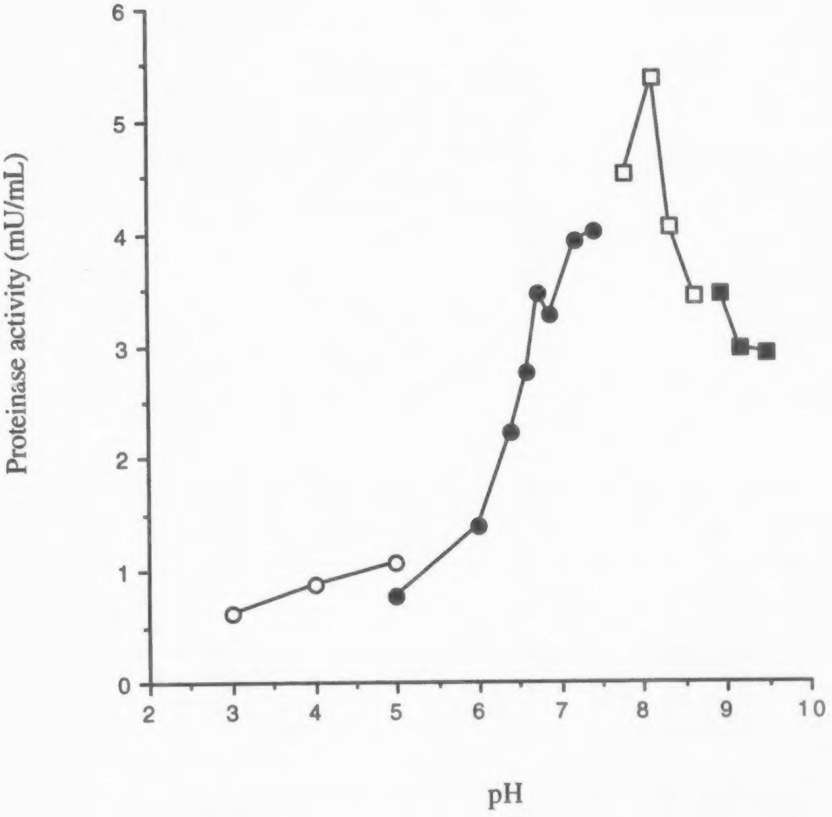
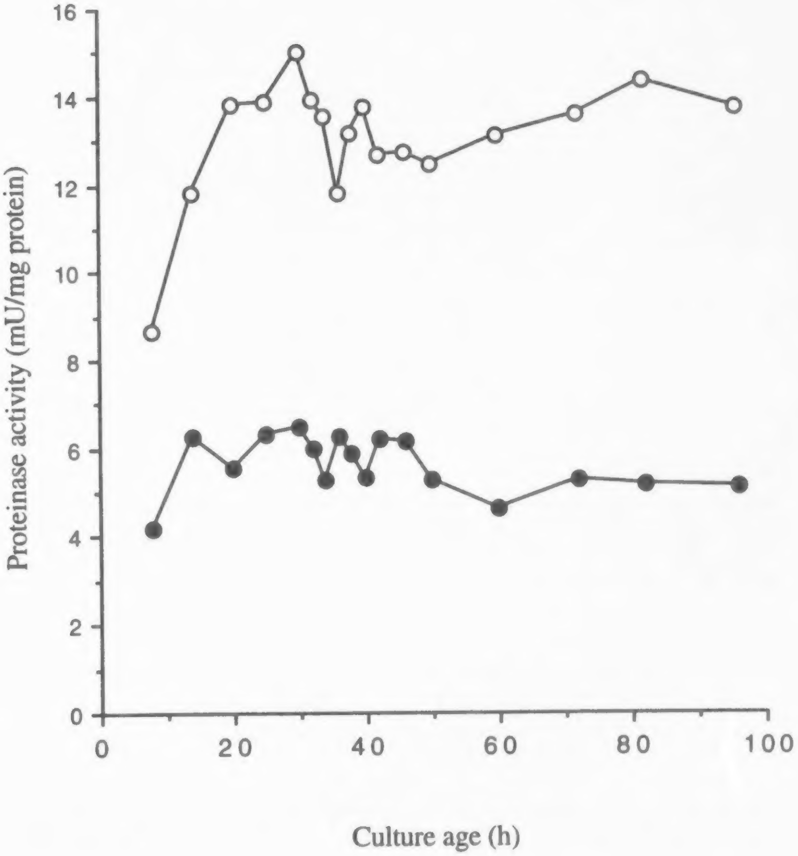

Proteinase activity (mU/mL)
0
1
2
3
4
5
6
2
3
4
5
6
7
8
9
10
pH

Figure 3.8 Time-course profile of intracellular proteinase.

Freshly prepared crude cell-free extracts obtained from cultures grown for different periods of time were analyzed for proteinase activity (●), then the cell-free extracts were incubated at 4°C for a period of 70 h and proteinolytic activity was assayed again (○) after storage.


16
14
12
10
8
6
4
2
0
Proteinase activity (mU/mg protein)
0
20
40
60
80
100
Culture age (h)

azocaseinolytic activity from the enzyme preparation no significant enhancement of ACV-synthetase stability was observed. Therefore, no direct effect of proteinases on the stability of ACV-synthetase could be established from these studies: factors other than proteinases must be involved in the inactivation of ACV-synthetase during storage.

3.1.6 Effects of additives on the stability of ACV-synthetase during storage.

The stability of partially purified ACV-synthetase was analyzed by following loss of initial enzyme activity during storage in the presence of various additives, to define a stabilization cocktail that will enhance the lifetime of the enzyme during storage and operation. DTT was most important of the additives tested in stabilizing the enzyme (Table 3.2). The most effective stabilizing mixture contained glycerol, $MgCl_2$, and the three substrate amino acids, in addition to DTT. It enabled the enzyme to retain full activity after 48 h of storage at 4°C, while the control had lost 75% of its initial activity. At room temperature significant loss in activity was observed within a few hours of incubation in the absence of the additives. In the presence of these additives however, a slight improvement in the stability of the enzyme was achieved. A combination of the optimal cell harvest time and the stabilization cocktail produced an enzyme preparation which retained more than 50% of its initial activity after 6.5 days of storage at 4°C. Storage of the enzyme at -75°C in the stabilization cocktail enabled retention of most of the initial activity over a period of several months.

3.2 Analysis of the effect of ACV-synthetase related protein (ACVSR) on the catalytic activity of ACV-synthetase.

Earlier enzyme purification studies presented evidence suggesting that ACV-synthetase existed as an heteropolymer, in association with a 32-kDa polypeptide (Jensen *et al.*, 1990). From the work in progress in the laboratory, it was found that the gene

Table 3.2. Effects of additives on the stability of ACV-synthetase during storage at 4°C.

	% Residual ACV-synthetase activity (2)					
Additive(s) (1)	0 h	24 h	48 h	96 h	120 h	156 h
Control	100	69	25	3	0	0
AAA	101	60	27	0	0	0
Cys	99	67	23	1	0	0
Val	100	70	30	3	0	0
AAA+Cys+Val	112	32	12	0	0	0
$MgCl_2$	98	36	1	0	0	0
$MgCl_2$+AAA+Cys+Val	107	27	2	0	0	0
Glycerol	100	76	45	21	1	0
Glycerol+AAA+Cys+Val	110	42	3	0	0	0
Glycerol+$MgCl_2$+AAA+Cys+Val	102	35	9	6	0	0
DTT	103	107	87	45	13	0
DTT+AAA+Cys+Val	105.2	95	83	56	29	2
DTT+Glycerol+$MgCl_2$+AAA+Cys+Val	109	113	98	67	53	49

(1) The ammonium sulfate fraction of ACV-synthetase from a 44-h culture was preincubated at 4°C with one or more of the following additives: AAA (L-α-aminoadipate at 5 mM), Cys (L-cysteine at 1 mM), Val (L-valine at 5 mM), DTT at 2 mM, $MgCl_2$ at 3 mM, and glycerol at 20% (v/v) in 80 mM MOPS/KOH buffer pH 7.5.

(2) ACV-synthetase activity remaining after the indicated storage period was determined, and expressed relative to the initial activity of the control at time 0 h. Activities are averages of duplicate activity values from two independent experiments.

encoding this low MW protein was located in the proximity of the cephamycin biosynthetic gene cluster in *S. clavuligerus* (Aidoo, K. and S.E. Jensen, Personal communication). In addition, sequence analysis studies revealed some similarity between this protein and arginase, an enzyme that converts arginine to ornithine and believed to be involved in the biosynthesis of clavulanic acid (Penninckx *et al.*, 1974; Romero *et al.*, 1986). This low MW protein was therefore termed ACVSR, for ACV-synthetase related protein. On the basis of this information, ACVSR was presumed to exist as a complex with ACV-synthetase and affect the activity of this enzyme, thus cephamycin biosynthesis; or this protein was simply involved in the biosynthesis of clavulanic acid either directly or in a regulatory role. It was also assumed that attempts to immobilize ACV-synthetase would result in the co-immobilization of the two proteins. Therefore, it was decided to investigate the function of ACVSR, most importantly its effect on ACV-synthetase activity. Two approaches were adopted. The first was to carry out physiological studies aiming at separating the clavulanic acid and cephamycin biosynthetic pathways during growth of *S. clavuligerus* in the presence of arginine, glutamate or ornithine according to Romero *et al.* (1986), and then analyze the effect on the activity, production and possible association of ACV-synthetase and ACVSR. The second approach was to attempt the separation of the two proteins *in vitro* through additional chromatographic purification steps, and then investigate the effect of ACVSR on ACV-synthetase.

3.2.1 Effects of arginine, glutamic acid and ornithine on ACV-synthetase activity, and on the production of clavulanic acid and antibiotics by cultures of *S. clavuligerus*.

Initial experiments investigated the effects on total antibiotic and clavulanic acid production by arginine, glutamic acid and ornithine, supplemented at 10 mM each to the starch plus asparagine-based medium (Aharonowitz and Demain, 1977), and to the glycerol plus proline-based medium (Romero *et al.*, 1986). The results indicated that ornithine inhibited the total antibiotic production in the glycerol plus proline-based medium (Fig. 3.9) as well as in the starch plus asparagine-based medium (not shown). However, in starch plus asparagine-based media arginine and glutamate supported good production of antibiotic, reaching total antibiotic titres of 80 to 100 μg/mL, as was the case in their absence in the control starch plus asparagine-based medium. No similar effect was observed with the glycerol plus proline-based media. Clavulanic acid production by cultures grown in glycerol plus proline-based media was highest with arginine, and followed by ornithine (Fig. 3.9).

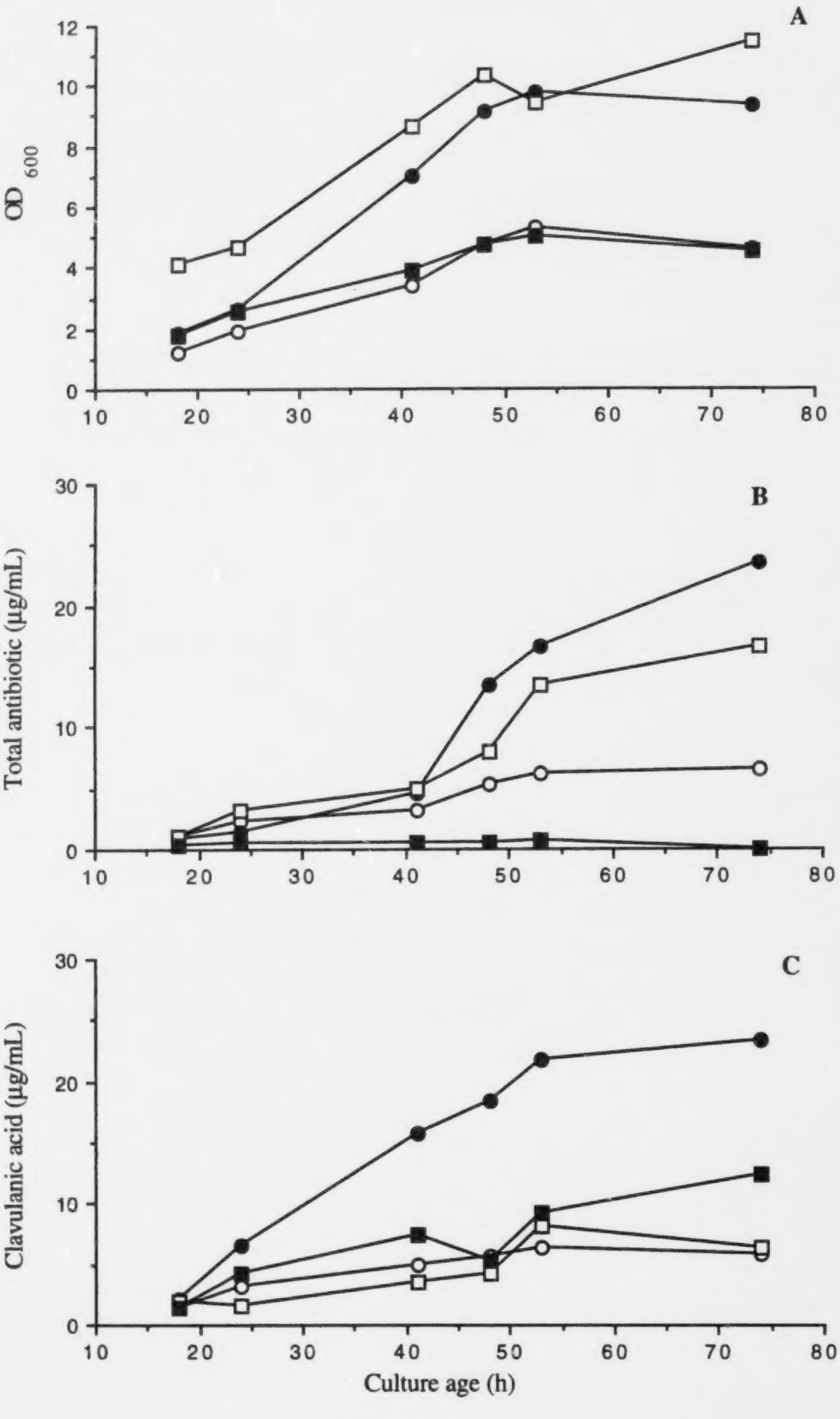
The effect of the three amino acids on ACV-synthetase and IPNS activities was also investigated with cell-free extracts from cultures grown in the glycerol plus proline-based media. ACV-synthetase and IPNS activities were highest in cultures grown in arginine-supplemented medium, followed by glutamate-supplemented medium and lowest in ornithine-supplemented medium. At concentrations of 1 mM and 10 mM, no activating or inhibiting effect by any of the three amino acids was observed on the catalytic activity of purified ACV-synthetase. However, new developments in this laboratory on ACVSR studies, combined with the inability to detect a direct effect by any of these amino acids on the catalytic activity of ACV-synthetase, suggested that this project was not a productive line of research at this time. Therefore, further experimentation attempting to determine the effect of growth in amino acid supplemented media on ACV-synthetase and ACVSR differential expression and related studies were suspended.

3.2.2 *In vitro* separation of ACVSR and ACV-synthetase, and analysis of the effect of purified ACVSR on ACV-synthetase activity.

Nevertheless, the question of the effect of ACVSR on the catalytic activity of ACV-synthetase was addressed by looking for unusual reaction products in ACV-synthetase reaction mixtures from the utilization of radioactively-labelled ATP. The expected products from α-^{32}P-ATP are α-^{32}P-AMP and PP_i, and from γ-^{32}P-ATP are

Figure 3.9 Effects of arginine, glutamic acid and ornithine on the production of cephamycin C and clavulanic acid by *S. clavuligerus* in glycerol plus proline-based medium.

A, Growth; **B**, total antibiotic; and **C**, clavulanic acid, in the glycerol plus proline-based growth medium (○), supplemented with arginine (●), glutamate (□), or ornithine (■), at 10 mM each.


A
OD $_{600}$
0
2
4
6
8
10
12
B
Total antibiotic (µg/mL)
0
10
20
30
C
Clavulanic acid (µg/mL)
0
10
20
30
10
20
30
40
50
60
70
80
Culture age (h)

AMP and ^{32}P-PP$_i$, based on the postulated amino acid activation and thioesterification activities of ACV-synthetase (Baldwin and Schofield, 1992) according to this scheme:

Reaction step 3.1: **α-^{32}P-ATP + a.a + E <----> E~a.a–α-^{32}P-AMP + PP$_i$** then,

Reaction step 3.2: **E~a.a–α-^{32}P-AMP <-----> E-a.a + α-^{32}P-AMP** or,

Reaction step 3.3: **γ-^{32}P-ATP + a.a + E <-----> E~a.a–AMP + ^{32}P-PP$_i$** then,

Reaction step 3.4: **E~a.a–AMP <------> E–a.a + AMP**

where **a.a** represents the amino acid, **E~a.a** and **E–a.a**, represent the enzyme-bound activated amino acid and enzyme-thioester linked amino acid intermediates, respectively. The purified ACV-synthetase plus ACVSR preparation was incubated in modified assay mixtures of ACV-synthetase in the presence of γ-^{32}P-ATP. The reaction mixtures were then analyzed by TLC on PEI-cellulose, and radioactive products and unreacted substrate visualized by autoradiography (Fig. 3.10). Panel A shows the enzyme controls: active enzyme (lane 1), enzyme whose activity has been lost during an overnight incubation at room temperature (lane 2), and the radioactive reagent controls: γ-^{32}P-ATP (lane 3) and heated γ-^{32}P-ATP (lane 4). Heating of γ-^{32}P-ATP was done to generate standards of P$_i$ and PP$_i$ from the hydrolysis of the phosphoester bonds (Dr. K.L. Roy, personal communication). The results showed that only a trace amount of radioactive PP$_i$ formed from γ-^{32}P-ATP in the presence of active enzyme (lane 1), this against some background amounts of ^{32}P-PP$_i$ as seen in the negative control (lane 3). Most unexpected was the formation of ^{32}P-ADP, observed predominantly in lane 1, suggesting that the formation of this product was dependent on an active enzyme preparation. The results in panel B indicated that ^{32}P-ADP formation was also dependent on the presence of the substrate amino acids of ACV-synthetase (lanes 1, 2 and 3). However, no ^{32}P-PP$_i$ could be detected except in the presence of PEP and Ca^{+2} (lanes 3), that appeared to stimulate the formation of this product. As was stated earlier, PEP and Ca^{+2} are two effectors of ACV-synthetase

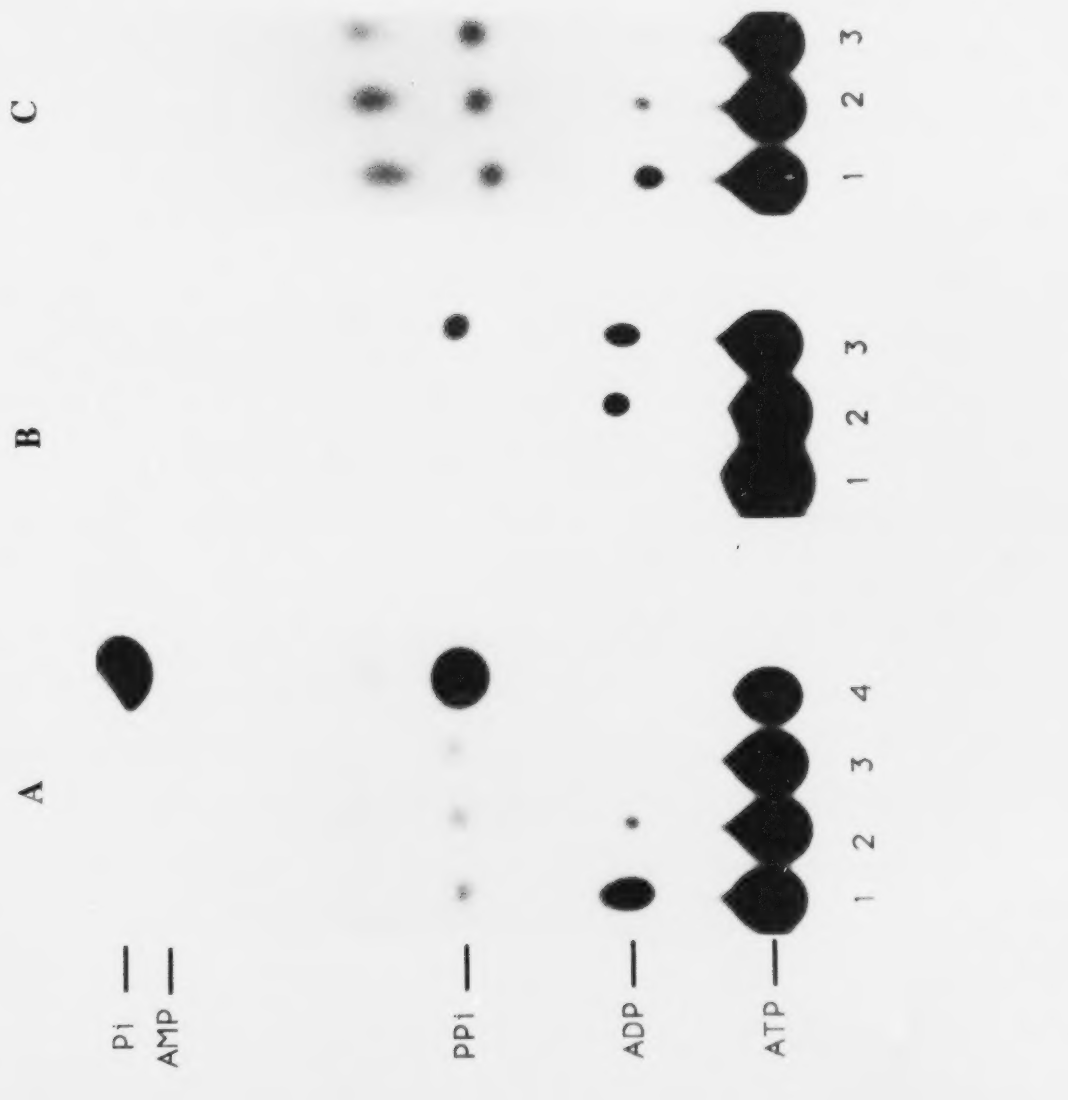
Figure 3.10 Thin layer chromatographic analysis of ACVSR and ACV-synthetase reaction mixture products with labelled γ-^{32}P-ATP.

Reaction mixtures in a total of 30 μL contained enzyme (20 μL) obtained after 2 passages through a Mono Q column, γ-^{32}P-ATP (3 μCi, 4500 Ci/mmol), unlabelled ATP (0.5 mM), $MgCl_2$ (1.5 mM) and DTT (2 mM) in 100 mM Tris buffer, pH 8.5; except in Panel B, the γ-^{32}P-ATP controls were prepared in the buffer only. The reaction was at room temperature for 1 h (except in Panel A, for 2 h), and stopped by spotting 3 μL of each mixture on a PEI-cellulose sheet for TLC analysis and visualization of the radioactive components by autoradiography. Internal standard mixture (1 μL) of unlabelled ATP, ADP and AMP (1 mM each) was spotted with each mixture to help identify the nucleotides.

Panel A shows the controls: (**1**) reaction mixture with amino acids; (**2**) as in (**1**) but the enzyme had been inactivated by overnight incubation at room temperature; (**3**) γ-^{32}P-ATP; and (**4**) γ-^{32}P-ATP heated at 100°C for 2 to 5 min to generate PP_i and P_i standards.

Panel B consists of (**1**) reaction mixture; (**2**) reaction mixture with substrate amino acids; and (**3**) as in (**2**) with also Ca^{+2} (3 mM) and PEP (5 mM).

Panel C consists of partial reaction steps of ACV-synthetase with (**1**) reaction mixture with L-cysteine; (**2**) reaction mixture with L-valine; and (**3**) reaction mixture with L-AAA.


A
B
C
Pi
AMP
PPi
ADP
ATP
1
2
3
4
1
2
3
1
2
3

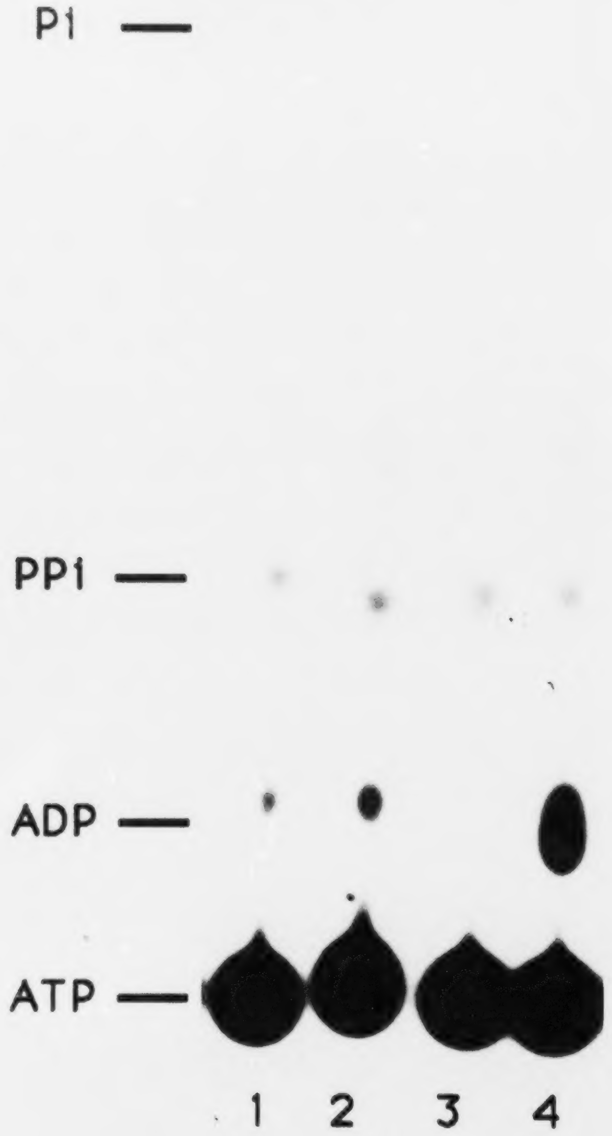
activity in partially purified enzyme preparations. Panel C illustrated the effect of individual substrate amino acid on product formation from γ-^{32}P-ATP, without ACV formation. The results showed a variation in the amount of ^{32}P-ADP formed with the type of amino acid added to the reaction mixtures. Qualitative analysis revealed that more ^{32}P-ADP was formed in the presence of cysteine (lane 1), slightly less with valine (lane 2), and at hardly detectable levels in the presence of L-AAA alone (lane 3). Similar observations have been made with ^{32}P-ATP formation in the ATP/^{32}P-PP$_i$ exchange reaction with these three amino acids (van Liempt *et al.*, 1989; Schwecke *et al.*, 1992). The formation of ^{32}P-ADP from γ-^{32}P-ATP was dependent on the activity of ACV-synthetase. The unidentified spots above PP$_i$ in panel C were also present in other panels but could not be seen because of the shorter exposure time used in the making of the photographs of panels A and B.

Subsequent studies using both γ-^{32}P-ATP indicated that the formation of ^{32}P-ADP in reaction mixtures supplemented with unlabelled AMP occurred in the absence of amino acids, but was stimulated by their presence and was time-dependent (Fig. 3.11). In reaction mixtures supplemented with unlabelled ADP, ^{32}P-ADP formation was greatly stimulated to much higher levels than those achieved with AMP supplementation but only in the presence of amino acids. This activity was also found to be time-dependent. These observations suggested that this activity was not likely an adenylate kinase whose reaction has an equilibrium constant of approximately 1 and is freely reversible (Noda, 1973). To establish that the observed activity generating ^{32}P-ADP from ^{32}P-ATP was not a novel function of ACV-synthetase or required the two proteins, attempts were made to separate ACVSR from ACV-synthetase.

The separation of the two proteins has been achieved by Zhang and Demain (1990a,b) who reported on the purification of ACV-synthetase to electrophoretic homogeneity, and by others (Schwecke *et al.*, 1992; Baldwin *et al.*, 1991). It was expected that additional purification steps such as gel exclusion and hydrophobic interaction chromatographies, which were omitted from our purification scheme (Jensen *et al.*, 1990),

Figure 3.11 Effect of AMP and ADP on the formation of labelled ADP from γ-^{32}P-ATP by ACVSR/ACV-synthetase preparation.

Reaction mixtures and sample treatments were as described in the legend to Figure 3.10. The assay conditions in each lane consisted of (**1**) reaction mixture with unlabelled AMP but without substrate amino acids; (**2**) reaction mixture with unlabelled AMP and substrate amino acids; (**3**) reaction mixture with unlabelled ADP without substrate amino acids; (**4**) reaction mixture with unlabelled ADP and substrate amino acids. ADP and AMP were used at 5 mM each.


Pi
PPi
ADP
ATP
1
2
3
4

would allow the separation of the two proteins. At this time, a parallel research project in the group showed that ACVSR was an octameric protein with a MW of about 240 kDa, and could be separated from ACV-synthetase by gel filtration. Figure 3.12 shows 5% and 10% SDS-PAGE profiles of Mono Q purified ACV-synthetase fractions. In the 5% SDS gel, ACV-synthetase was apparently free of contaminating proteins. The 10% SDS gel, however, showed another protein band of about 32 kDa, corresponding to ACVSR. Application of the Mono Q purified enzyme to a Superose 6B gel exclusion column resulted in the separation of the two proteins as seen in panel C.

With purified proteins, it was found that the ^{32}P-AMP forming activity was associated with ACV-synthetase and the ^{32}P-ADP forming activity with ACVSR fraction (Fig. 3.13). In this study, ACV-synthetase and ACVSR Superose 6B fractions were reacted separately or together under a variety of conditions in the presence of α-^{32}P-ATP. Also included in this study was the initial Mono Q fraction that was loaded on the gel filtration column. The same reaction mixtures, lacking the radioactively labelled ATP, were analyzed by HPLC for ACV-synthetase activity. ACV-synthetase activity was detected in the absence of ACVSR. When combined with ACVSR, ACV-synthetase activity was not affected. As a results no further studies were done to determine the function and activity of ACVSR and these were considered to be investigation topics for others.

3.3 Immobilization of *S. clavuligerus* ACV-synthetase and production of ACV by immobilized enzyme.

In a previous report, the partially purified ACV-synthetase from *S. clavuligerus* was immobilized on the anion-exchange resin DEAE-Trisacryl and was shown to carry out repeatedly the synthesis of ACV (Jensen *et al.*, 1989). However, after six uses the enzyme lost 70% of the initial activity and the proportion of product formed was quite low. The present studies investigated other immobilization methods and ways to improve both enzyme stability and product yields.

Figure 3.12 SDS-PAGE analysis of ACVSR and ACV-synthetase separated by gel exclusion chromatography.

A: 5% polyacrylamide gel of consecutive peak fractions of ACV-synthetase in lanes **1**, **2** and **3**, from a second run through a Mono Q column.

B: 10% polyacrylamide gel of the same fractions as in **A** in lanes **2**, **3**, **4** and standards in lane **1**.

C: 10% polyacrylamide gel. Lane **1** and **2** show ACV-synthetase and ACVSR, respectively, from the Superose 6B column; and lane **3** shows the enzyme pool fraction sample after the first Mono Q column, that was loaded on the Superose 6B column. The protein concentration loaded was in the range of 1 to 3 µg in each lane. The protein band locations for ACV-synthetase and ACVSR are indicated with arrows. Standard protein MWs are indicated on the left side in panels A and B for myosin (200 kDa), ß-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), hen egg ovalbumin (45 kDa), and bovine pancreas trypsinogen (25 kDa).

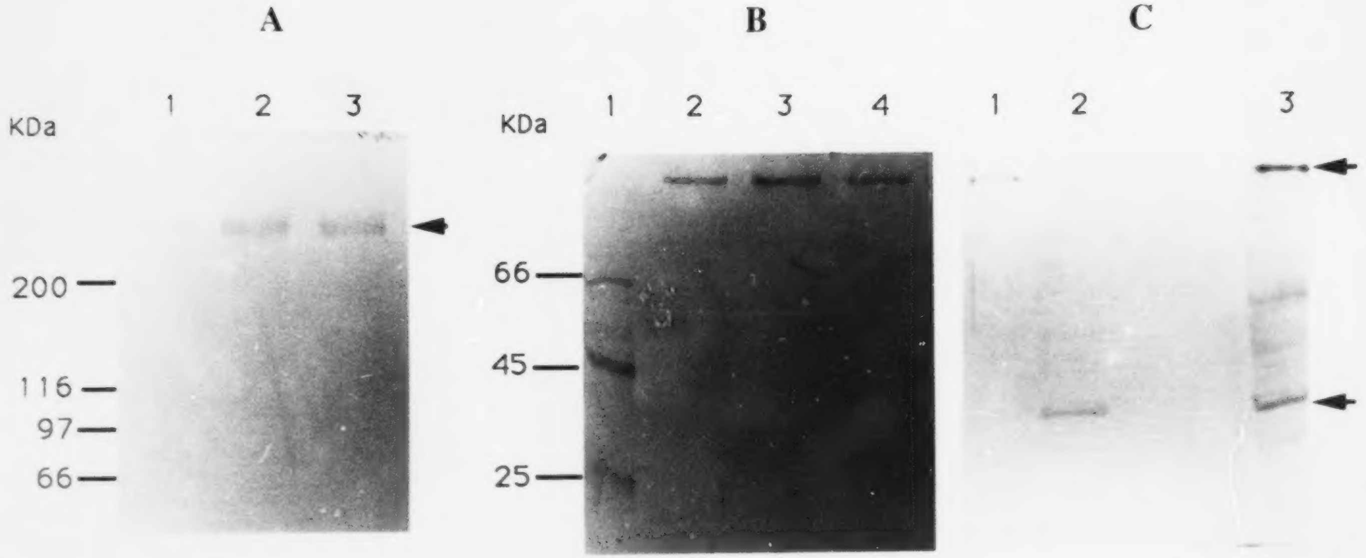

A
B
C
KDa
1
2
3
200
116
97
66
KDa
1
2
3
4
66
45
25
1
2
3

Figure 3.13 Effect of separation of ACVSR from ACV-synthetase activity: TLC analysis of the reaction products from α-^{32}P-ATP.

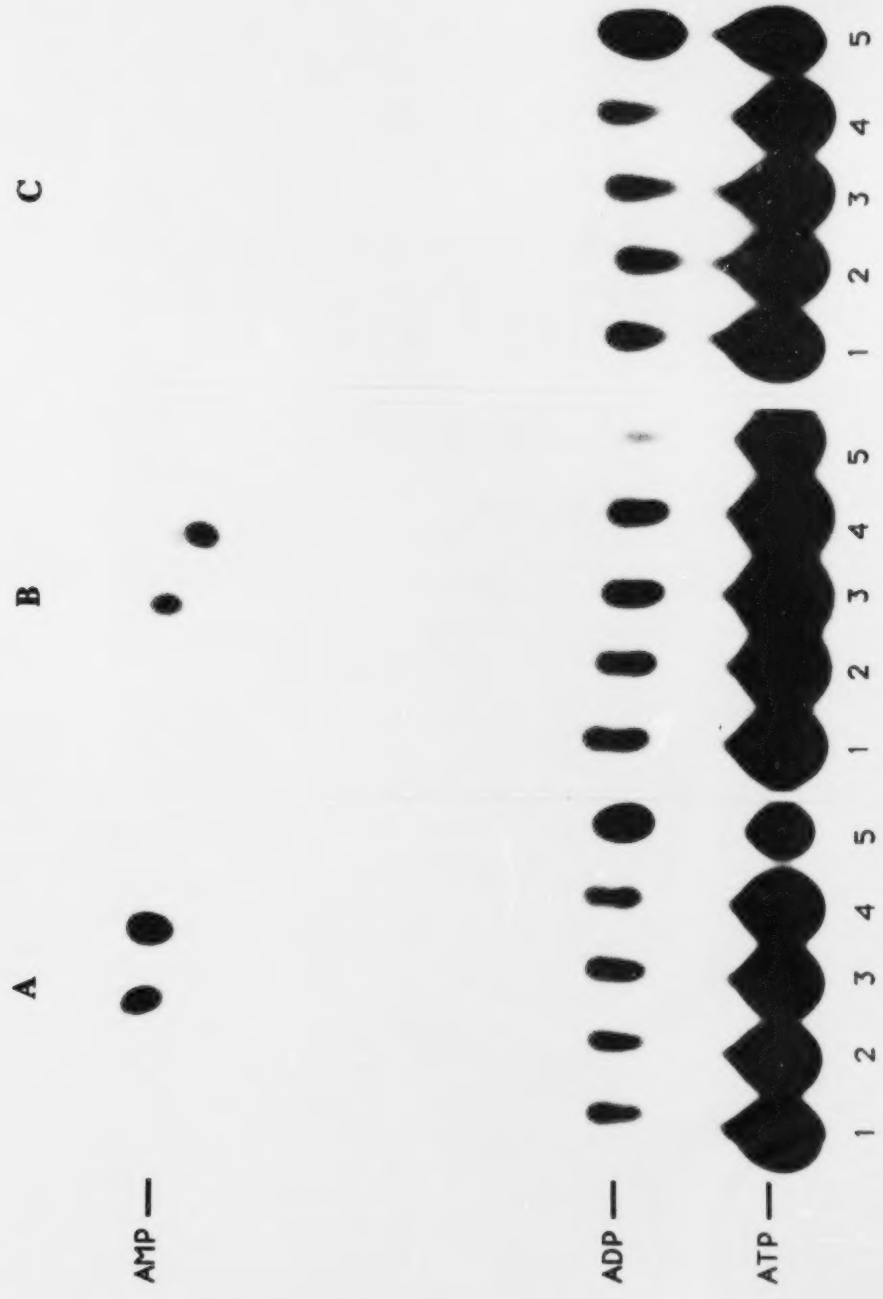
Basic reaction mixtures contained protein (20 μL) and concentrated reagents (10 μL), consisting of α-^{32}P-ATP (5 μCi, 3000 Ci/mmol)), unlabelled ATP (0.5 mM), $MgCl_2$ (3 mM), DTT (2 mM) and the substrate amino acids, up to 30 μL in 100 mM Tris/HCl buffer, pH 8.5. The reaction was for 1 h at room temperature and the samples were treated as described in the legend to Figure 3.10.

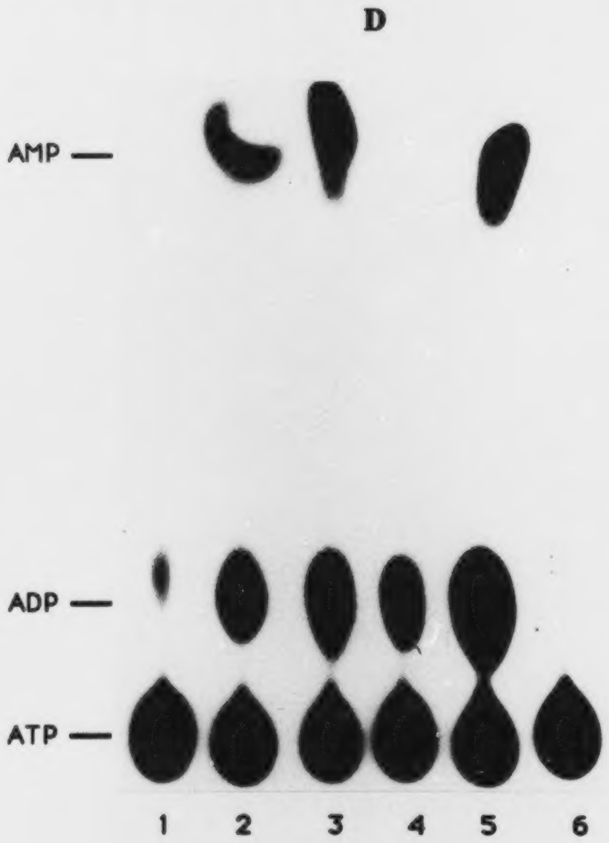
Panel A: reconstituted mixture of ACVSR and ACV-synthetase from purified proteins (10 μL each of concentrate) from the Superose 6B column.

Panel B: purified ACV-synthetase only (20 μL of concentrate) from the Superose 6B column.

Panel C: purified ACVSR only (20 μL each of concentrate) from the Superose 6B column.

Panel D: Co-purified ACVSR and ACV-synthetase proteins (20 μL of concentrate) from the Mono Q column, that was loaded onto the Superose column. The reaction conditions consisted of the basic mixture without substrate amino acids (lane **1**), with substrate amino acids (lane **2**), with substrate amino acids and PEP (5 mM) plus Ca^{+2} (3 mM) (lane **3**), with substrate amino acids and AMP (5 mM) (lane **4**), with substrate amino acids and ADP (5 mM) (lane **5**). The additional lane **6** in panel D is the control reaction α-^{32}P-ATP only. The ADP spots observed in lanes 1 to 4 is mainly due the background radioactivity resulting from the chemical degradation of α-^{32}P-ATP, and was also observed in the negative control (not shown). In lane 5, enzyme-dependent ADP formation can be observed against this background level.


A
B
C
AMP
ADP
ATP
1
2
3
4
5
1
2
3
4
5
1
2
3
4
5


D
AMP
ADP
ATP
1
2
3
4
5
6

3.3.1 Immobilization of ACV-synthetase by covalent bonding to aminopropyl controlled-pore glass.

Attempts were made initially to covalently immobilize ACV-synthetase onto porous glass beads (240-Å pore size, 200-400 mesh size), using two methods. In the first method (Weetall 1976) the enzyme was linked to aminopropyl glass with glutaraldehyde. In the second, a modification of the method of Weetall (Kadima and Pickard, 1990), the enzyme was first ionically bound onto aminopropyl glass, and then crosslinked with glutaraldehyde under low ionic strength conditions.

The conditions for the immobilization of ACV-synthetase on aminopropyl glass by the modified method were first established by analyzing the effect of pH on the coupling yield and stability of the enzyme. The effect of pH on enzyme stability was tested from pH 5 to 9 during an overnight storage at -75°C and 4°C. The enzyme was most stable at pH 7.5 at both temperatures, but the results indicated also a decrease in the activity of samples stored at 4°C, in comparison to those stored at -75°C (Fig. 3.14). The enzyme was extremely unstable at pH 5.0, where no detectable activity was observed. Upon addition to the buffer system at pH 5, the enzyme solution turned turbid, presumably due to protein denaturation. Analysis of the effect of pH on the coupling yield of ACV-synthetase onto aminopropyl glass indicated optimal binding at pH 8.0 (Fig. 3.15A). However, when taking into consideration the stability of the enzyme at each pH tested, and estimating the level of bound ACV-synthetase protein on the basis of residual activity of the corresponding soluble enzyme control incubated at 4°C, it was evident that binding of the enzyme occurred also at pH 9, despite its instability at this pH (Fig. 3.15B).

The covalent immobilization of partially purified ACV-synthetase was then carried out by incubating the enzyme (7.8 mg/mL protein, 0.13 mU/mg) with aminopropyl controlled pore glass (CPG, 0.1 mg dry weight), and the same amount of enzyme was added to another aminopropyl CPG that was previously treated with glutaraldehyde. The mixtures were incubated at 4°C for 6 h, then the samples were washed with low ionic

Figure 3.14 pH effect on the stability of ACV-synthetase during storage.

Partially purified enzyme solutions (9.2 mg protein/mL, 0.218 mU/mg) were prepared in 10 mM buffers containing 20% glycerol and 1 mM DTT, at pH values ranging from 5 to 9, using a set of pre-equilibrated desalting columns. Initial activity was determined and the enzyme solutions were divided into two: one incubated at 4°C (■) and the other frozen at -75°C (▨). After an overnight-storage, the residual activity was determined in the standard ACV-synthetase assay at pH 8.5, and is expressed as a percent relative to the initial activity of the enzyme prepared in the pH 7.5 buffer. The values are averages of duplicate data from two separate experiments.

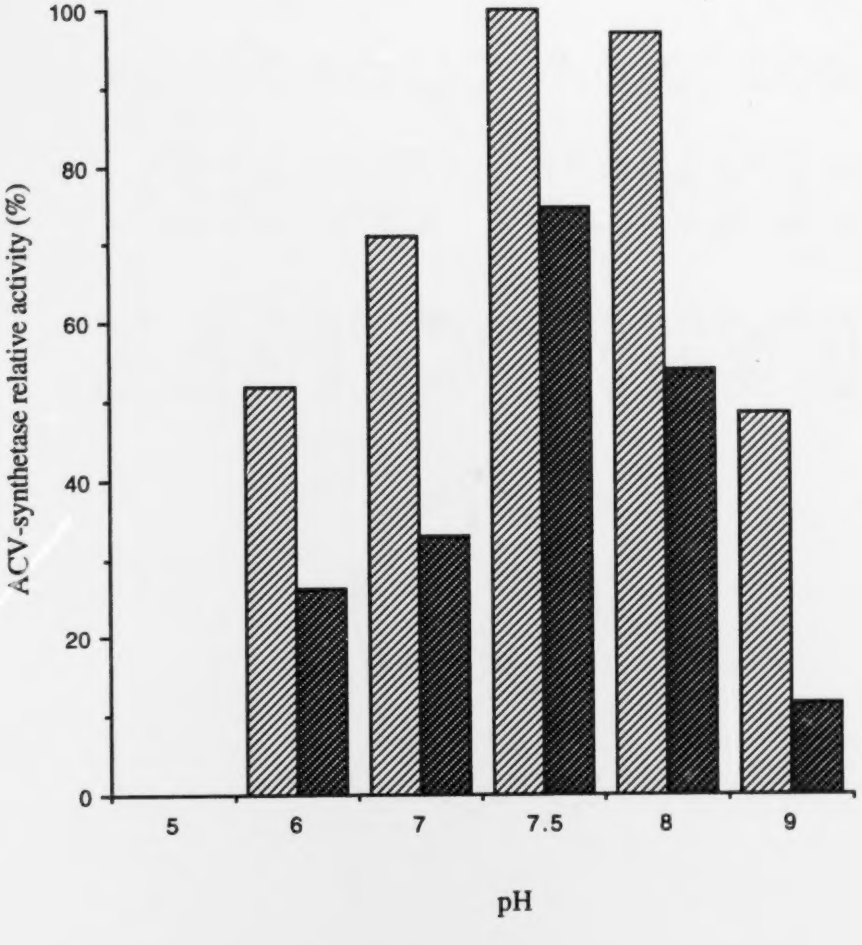
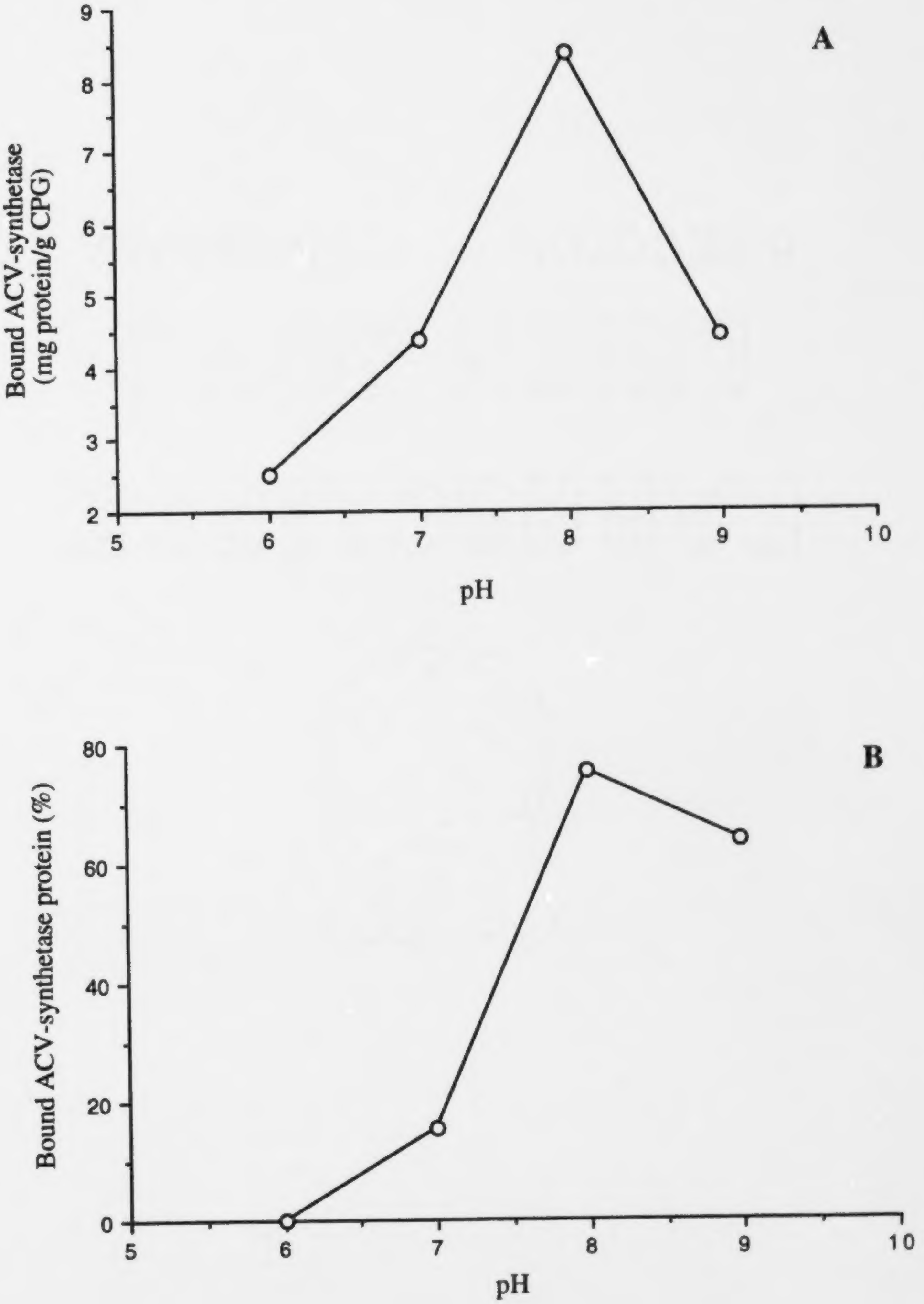

ACV-synthetase relative activity (%)
100
80
60
40
20
0
5
6
7
7.5
8
9
pH

Figure 3.15 pH effect on the ionic binding of ACV-synthetase to aminopropyl glass. Partially purified enzyme solutions (9.2 mg protein/mL, 0.218 mU/mg) were prepared as described in the legend to Figure 3.14. Each enzyme solution (0.5 mL) was reacted with aminopropyl glass (0.1 mg dry weight) at 4°C. A control set of soluble enzyme preparation at each pH was also incubated at 4°C. After an overnight incubation, unbound enzyme was measured as combined residual activity in solution and from the washings. Bound ACV-synthetase was defined as the fraction of enzyme activity removed from the soluble phase.

A: The total amount of bound ACV-synthetase.

B: The net amount of bound ACV-synthetase obtained by substracting from (**A**) the loss of activity due to incubation of the soluble enzyme at 4°C without aminopropyl glass.


A
Bound ACV-synthetase (mg protein/g CPG)
9
8
7
6
5
4
3
2
5
6
7
8
9
10
pH
B
Bound ACV-synthetase protein (%)
80
60
40
20
0
5
6
7
8
9
10
pH

strength stabilization solution of ACV-synthetase. To one of the two samples not treated with glutaraldehyde, a solution of glutaraldehyde (1 mL, 2.5%) was added and incubated for 1 h at room temperature to allow the crosslinking of the protein ionically bound to the aminopropyl glass. The two samples of covalently bound proteins were subsequently washed with high ionic strength buffer (0.1 M MDG buffer, pH 7.5 containing 0.5 M NaCl). The resulting products were analyzed for activity, and in both cases no detectable catalytic activity was observed.

3.3.2 Immobilization of ACV-synthetase by ionic bonding to aminopropyl controlled-pore glass and DEAE-Trisacryl.

An alternative immobilization method was considered, involving the binding of ACV-synthetase to porous glass and DEAE-Trisacryl resin following the methods of Kadima and Pickard (1990) and Jensen *et al.*, (1989), respectively. From a 2-L culture (2.6 g protein, 0.05 mU/mg), a Mono Q purified ACV-synthetase solution (11.1 mg protein, 0.64 mU/mg) was prepared. The enzyme was desalted in a series of dilution and concentration steps in 20 mM MOPS buffer, pH 7.5, containing the enzyme stabilization cocktail (20% glycerol, 5 mM DTT, 3 mM $MgCl_2$, 5 mM L-AAA, 1 mM Cysteine and 5 mM valine) to reduce the ionic strength of the protein sample prior to immobilization. Half of the sample was loaded in a 1-mL syringe containing 0.5 g of damp DEAE-Trisacryl resin and the other half used for immobilization on 0.1 g of aminopropyl porous glass, contained also in a 1-mL syringe. After the 2-mL protein sample was circulated 3 times through the carrier, and unbound protein removed with washing with low ionic strength stabilization buffer (4 mL). It was found that all of the initial protein in solution bound to DEAE-Trisacryl but only 55% bound to aminopropyl glass.

Subsequent studies analyzed the activity and stability of ACV-synthetase immobilized onto DEAE-Trisacryl and aminopropyl glass in a batch reactor with shaking at room temperature. The efficiencies of the two immobilized enzyme systems were determined by following the rate of ACV formation during a 1-h operation. A significant activity was observed with ACV-synthetase immobilized on DEAE-Trisacryl, which catalyzed the formation of ACV at 4.4% of the rate of the soluble enzyme; whereas the specific activity of the aminated-CPG bound enzyme was only 1.7% of the soluble enzyme (Table 3.3). Further studies with the DEAE-Trisacryl immobilized enzyme indicated that increasing the shaking speed resulted in a slight increase in the activity of the immobilized enzyme to 6.3% of the activity of the soluble enzyme (Table 3.4). When the formation of ACV was monitored with time, a linear rate of synthesis was observed for about 1.5 h then decreased markedly (Fig. 3.16). Supplementation of the rate limiting substrate cysteine to the reaction mixture did improve the production level, but only slightly. No significant increase in the level of ACV produced was observed when the reactor was left in operation overnight. Supplementation of fresh reagents to the reactor indicated a loss of catalytic activity of the immobilized enzyme reactor shaken overnight, yet the unshaken reactor control system retained a fraction of its initial activity.

These results implied that shaking was good for improving mass transfer of the system but the resulting increase in aeration destabilized the enzyme (see section 3.3.3 below). In addition, the low specific activity of the immobilized enzyme was less than desired for production of large quantities of ACV, unless a significant improvement in the lifetime of the reactor was achieved. As a result, a third procedure was considered which consisted of physically confining the enzyme in a porous filtration unit.

3.3.3 Immobilization of ACV-synthetase by physical confinement within porous filtration unit.

Confinement of ACV-synthetase was carried out in A 50-mL Amicon filtration unit using a 30-50 kDa-cutoff membrane. The enzyme used was the 35-45% saturation ammonium sulfate fraction of a crude *S. clavuligerus* extract, desalted by ultrafiltration

Table 3.3 Activity of ACV-synthetase immobilized by ionic bonding onto DEAE-Trisacryl and aminopropyl CPG.

Support	Total bound protein(1) (mg)	Total bound activity(1) (mU)	Observed total bound activity (mU)	Observed bound specific activity (mU/mg)	Total bound protein(1) (%)	Immobilized ACV-synthetase activity(2) (%)
DEAE-Trisacryl (0.5 g wet weight)	5.45	3.52	0.154	0.028	98.3	4.4
aminopropyl CPG (0.1 g dry weight)	3.08	1.12	0.033	0.011	55.5	1.7

(1) Estimated by difference from unbound protein or activity

(2) % of equivalent specific activity of the soluble enzyme of 0.64 mU/mg protein.

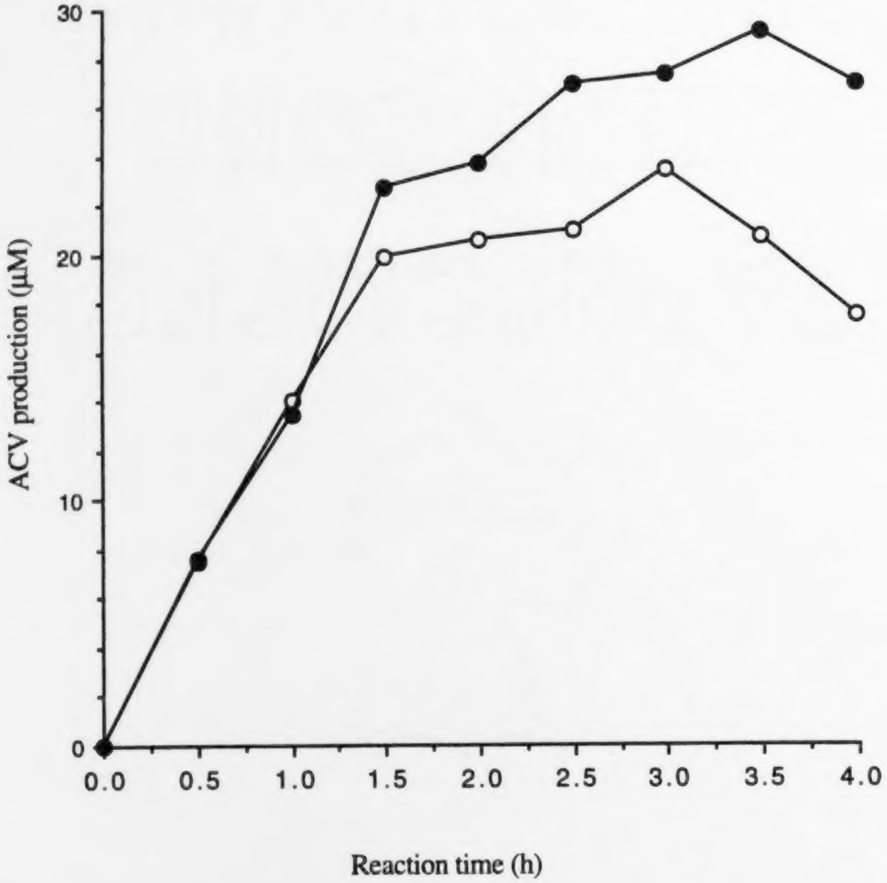
Table 3.4 Effect of shaking on the activity of ACV-synthetase immobilized by ionic bonding onto DEAE-Trisacryl

Shaker speed (rpm)	(1) Immobilized ACV-synthetase activity (%)
188	4.4
278	6.3

(1) % of equivalent specific activity of the soluble enzyme of 0.64 mU/mg protein.

Figure 3.16 Time-course of ACV production by ACV-synthetase immobilized onto DEAE-Trisacryl and the effect of L-cysteine supplementation.

A 4-mL solution of a Mono Q purified ACV-synthetase (11.1 mg protein, 0.64 mU/mg) was divided into two portions and each sample reacted with 0.5 g of damp DEAE-Trisacryl resin loaded in a 1-mL syringe. The enzyme solution was circulated 3 times through the carrier, and washed with low ionic strength ACV-synthetase stabilization solution (4 mL) to remove unbound protein. The immobilized enzyme materials were placed in two conical-bottom screw-cap bottles (2 mL in size) to which a 1 mL of the standard assay mixture was added. The samples were shaken at 188 rpm at room temperature for 4 h. To one sample, 5 μL of concentrated cysteine was added incrementally to a final concentration of 0.1 mM each time at 30-min intervals (●) and the other sample served as the unsupplemented control (○). At 30-min intervals, aliquots (20 μL) of the reaction mixture were removed and assayed for ACV production.



30
20
10
0
ACV production (µM)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
Reaction time (h)

using an X-300 membrane. This procedure also achieved a further purification of the enzyme.

3.3.3.1 Optimization of the synthesis of ACV by ACV-synthetase physically confined within porous filtration unit.

In studies for ACV production, the focus shifted from optimizing the initial catalytic rate to optimizing the overall substrate conversion. The effects of substrates on ACV-synthetase activity was studied with purified enzyme and the Michaelis constants determined. ACV-synthetase had the highest affinity for L-cysteine. The Km values for the three substrate amino acids were: 6.25, 0.43, 3.75 x 10^{-4} M for L-AAA, L-cysteine and L-valine, respectively (Figs. 3.17, 3.18 and 3.19). L-cysteine was found to be inhibitory at higher concentrations, and a similar observation was made with ATP (Fig. 3.20). A Michaelis constant value of 7.58 x 10^{-4} M was determined for ATP.

Since studies on the optimization of storage stability of ACV-synthetase were carried out primarily at 4°C and the enzyme demonstrated greater stability at this temperature than at room temperature, the possibility of operating the immobilized enzyme reactor at a lower temperature was envisaged. Analysis of the rate of catalysis of the purified enzyme over a temperature range of 5 to 24°C indicated an increase of ACV-synthetase activity in a linear fashion, with an estimated Q_{10} value of 2 (Fig. 3.21). However, the slow rate of catalysis at lower temperatures obviated the use of the enzyme at such temperatures, since the conditions for the most effective operating temperature, taking into account enzyme stability, were not analyzed.

ACV-synthetase is believed to have sulfhydryl groups essential for its activity (van Liempt *et al.*, 1989; Zhang *et al.*, 1992), that are subject to oxidation by oxygen in the atmosphere. The effect of the removal of oxygen from the reactor was investigated, by comparing activity in air and nitrogen atmospheres, to determine the stability of the enzyme during operation. The results in Table 3.5 suggested that the enzyme was more stable in a

Figure 3.17 Determination of the Michaelis constant of ACV-synthetase for L-α-aminoadipate.

The initial rate of ACV formation by purified ACV-synthetase (0.07 mg protein) was measured using initial concentrations of L-α-aminoadipate from 0.1 to 20 mM in the assay mixture. The initial concentrations of all other reagents were kept constant at: ATP (2 mM), $MgCl_2$ (15 mM), DTT (2 mM), L-cysteine (1 mM), and L-valine (5 mM) in 100 mM Tris/HCl buffer, pH 8.5.

A: The Michaelis-Menten plot of L-α-aminoadipate concentration vs ACV-synthetase activity. **B**: The double reciprocal plot.

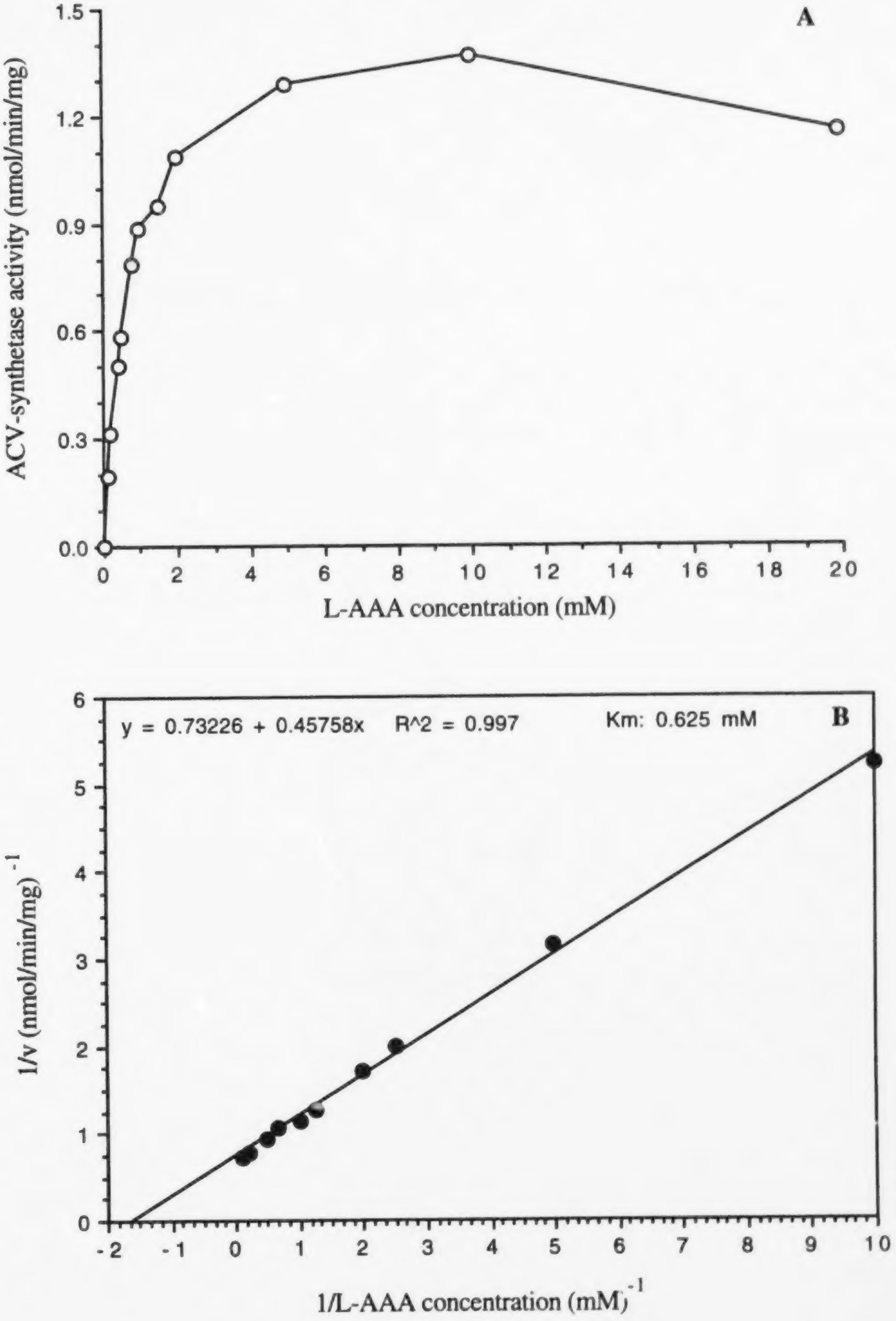

A
1.5
1.2
0.9
0.6
0.3
0.0
ACV-synthetase activity (nmol/min/mg)
0
2
4
6
8
10
12
14
16
18
20
L-AAA concentration (mM)
B
y = 0.73226 + 0.45758x R^2 = 0.997
Km: 0.625 mM
6
5
4
3
2
1
0
1/v (nmol/min/mg) -1
-2
-1
0
1
2
3
4
5
6
7
8
9
10
1/L-AAA concentration (mM) -1

Figure 3.18 Determination of the Michaelis constant of ACV-synthetase for L-cysteine.

The initial rate of ACV formation by purified ACV-synthetase (0.07 mg protein) was measured using initial concentrations of L-cysteine from 0.025 to 10 mM in the assay mixture. The initial concentrations of all other reagents were kept constant at: ATP (2 mM), $MgCl_2$ (15 mM), DTT (2 mM), L-α-aminoadipate (5 mM), and L-valine (5 mM) in 100 mM Tris/HCl buffer, pH 8.5.

A: The Michaelis-Menten plot of L-cysteine concentration vs ACV-synthetase activity. **B**: The double reciprocal plot.

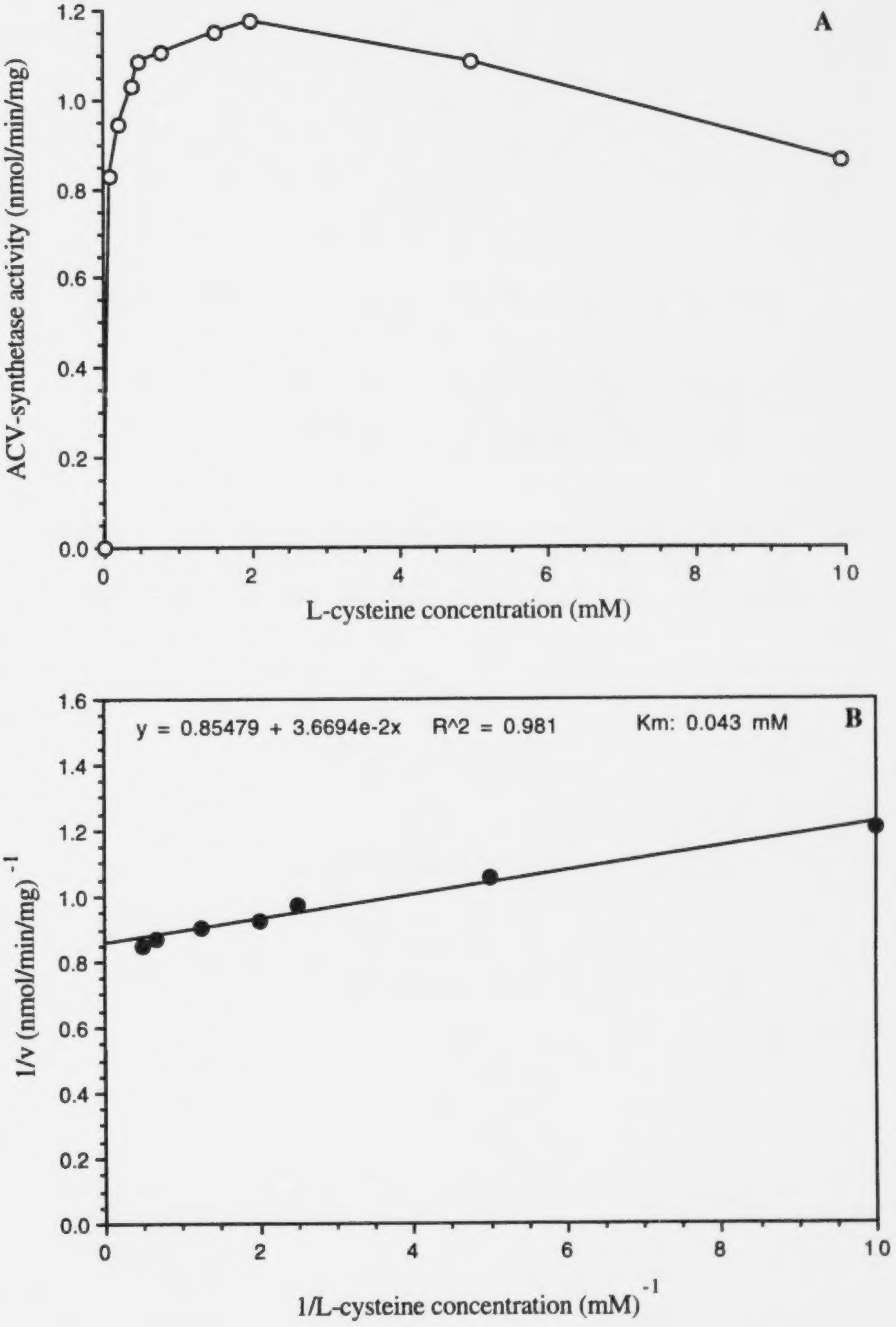

A
ACV-synthetase activity (nmol/min/mg)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
2
4
6
8
10
L-cysteine concentration (mM)
B
y = 0.85479 + 3.6694e-2x   R^2 = 0.981
Km: 0.043 mM
1/v (nmol/min/mg) -1
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0
2
4
6
8
10
1/L-cysteine concentration (mM) -1

Figure 3.19 Determination of the Michaelis constant of ACV-synthetase for L-valine. The initial rate of ACV formation by purified ACV-synthetase (0.07 mg protein) was measured using initial concentrations of L-valine from 0.1 to 20 mM in the assay mixture. The initial concentrations of all other reagents were kept constant at: ATP (2 mM), $MgCl_2$ (15 mM), DTT (2 mM), L-α-aminoadipate (5 mM), and L-cysteine (1 mM) in 100 mM Tris/HCl buffer, pH 8.5.

A: The Michaelis-Menten plot of L-valine concentration vs ACV-synthetase activity. **B**: The double reciprocal plot.

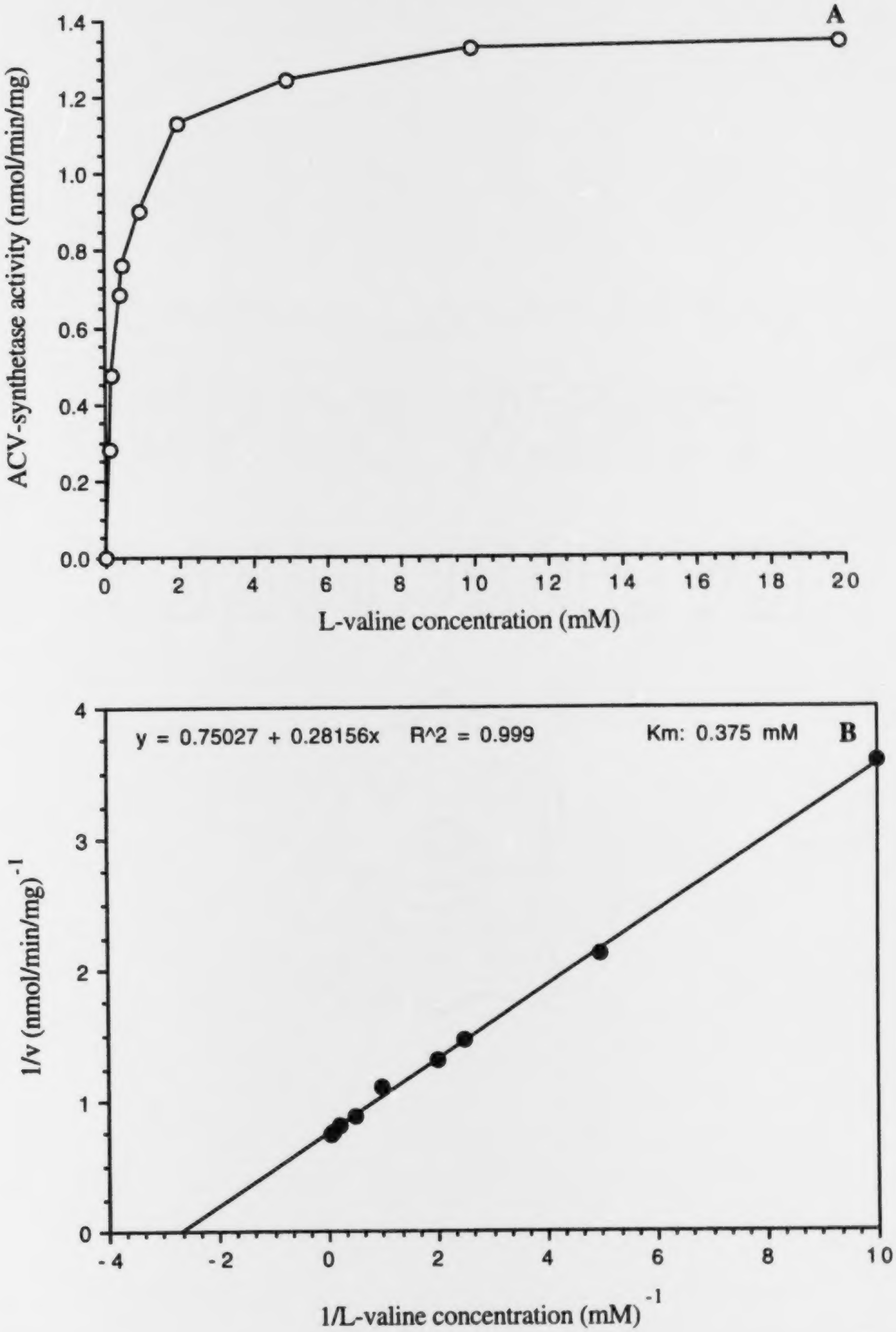
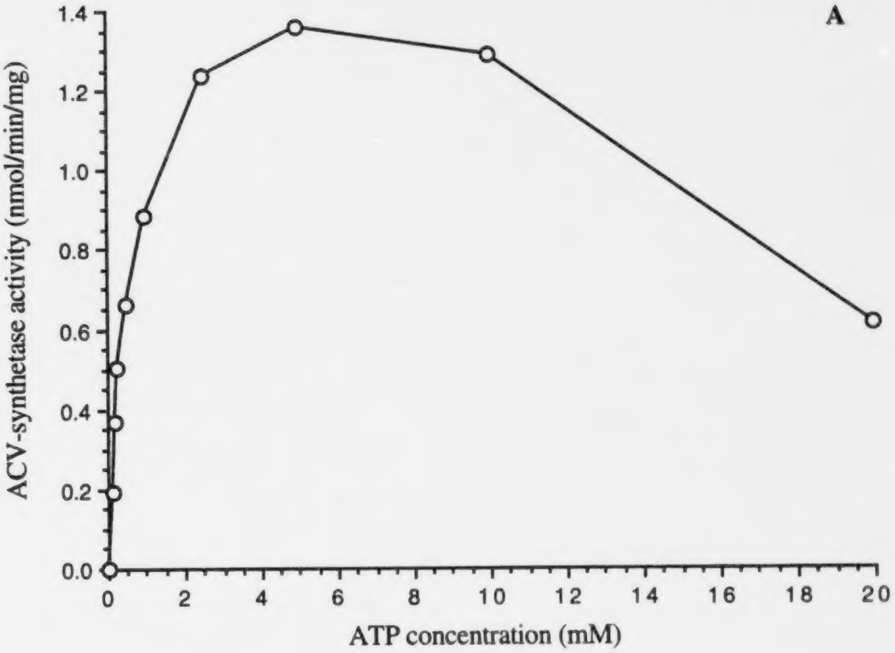
A
ACV-synthetase activity (nmol/min/mg)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
14
16
18
20
L-valine concentration (mM)
y = 0.75027 + 0.28156x   R^2 = 0.999
Km: 0.375 mM
B
1/v (nmol/min/mg)^-1
4
3
2
1
0
-4
-2
0
2
4
6
8
10
1/L-valine concentration (mM)^-1

Figure 3.20 Determination of the Michaelis constant of ACV-synthetase for ATP. The initial rate of ACV formation by purified ACV-synthetase (0.07 mg protein) was measured using initial concentrations of ATP from 0.1 to 20 mM in the assay mixture. The initial concentrations of all other reagents were kept constant at: $MgCl_2$ (15 mM), DTT (2 mM), L-α-aminoadipate (5 mM), and L-cysteine (1 mM) and L-valine (5 mM) in 100 mM Tris/HCl buffer, pH 8.5. **A:** The Michaelis-Menten plot of ATP concentration vs ACV-synthetase activity. **B**: The double reciprocal plot.


A
ACV-synthetase activity (nmol/min/mg)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
14
16
18
20
ATP concentration (mM)

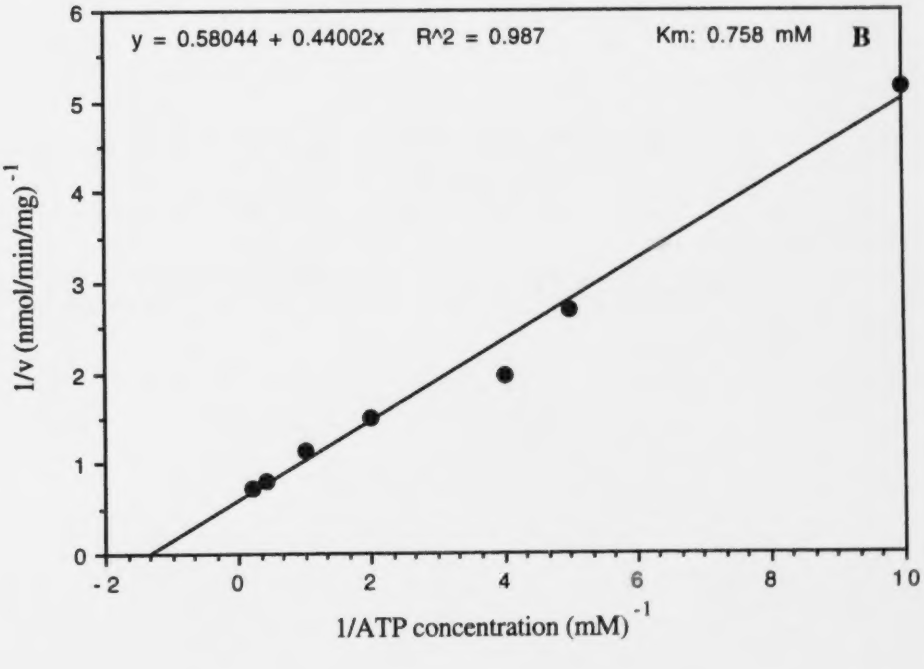

y = 0.58044 + 0.44002x R^2 = 0.987
Km: 0.758 mM
B
1/v (nmol/min/mg)$^{-1}$
6
5
4
3
2
1
0
-2
0
2
4
6
8
10
1/ATP concentration (mM)$^{-1}$

Figure 3.21 Effect of temperature on the catalytic rate of ACV-synthetase.

The initial rate of ACV formation by purified ACV-synthetase was measured at different temperature in the standard ATP (2 mM) PEP (5 mM) assay mixture.

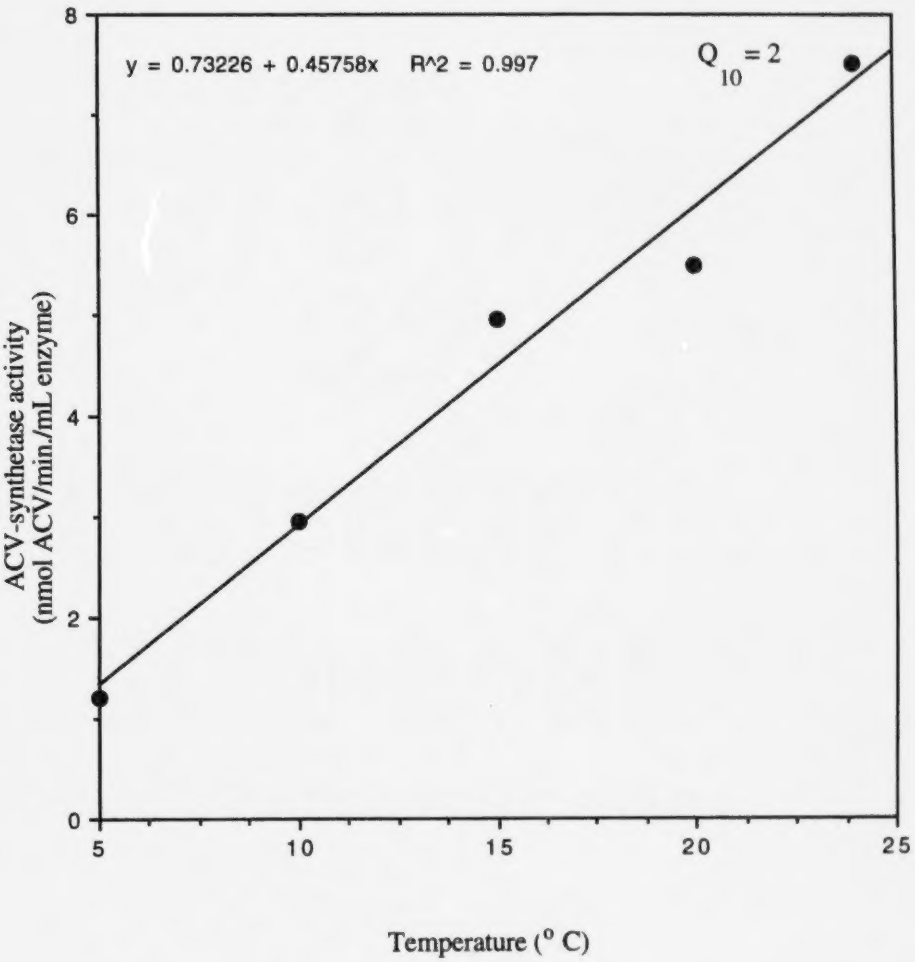

y = 0.73226 + 0.45758x R^2 = 0.997
$Q_{10} = 2$
ACV-synthetase activity
(nmol ACV/min./mL enzyme)
0
2
4
6
8
5
10
15
20
25
Temperature (° C)

Table 3.5 Effect of air and nitrogen atmospheres on the stability of ACV produced in the immobilized ACV-synthetase reactor.

	ACV produced (µg/mL)	
Condition (1)	1 h	16 h
Air	9.8	0.0
Air + DTT	13.3	26.4
Nitrogen	12.6	20.3
Nitrogen + DTT	10.9	20.3

(1) ACV production was carried out with partially purified enzyme in the presence of air or nitrogen atmosphere, at room temperature. At 1 h and 16 h a small amount of the mixture was removed by filtration from each reactor and divided in two 100-µL samples. For the conditions "Air" and "Nitrogen": the samples were directly derivatized with the thiolyte reagent for HPLC analysis of ACV. The conditions "Air + DTT" and "Nitrogen + DTT": the samples were first treated with DTT (1 mM) for 15 min prior to derivatization.

nitrogen atmosphere than air as shown later during repeated use studies. The results also indicated that the product, ACV, was much more stable under nitrogen atmosphere. However, the disappearance of ACV from the reaction mixture after prolonged (overnight) incubation at 22-24°C, in the presence of forced air, was reversed by adding DTT to the reaction mixture at the end of the incubation period. This piece of data suggests that ACV, like ACV-synthetase, was subject to air oxidation, and a nitrogen atmosphere was essential for their stability.

3.3.3.2 Semi-continuous production of ACV by ACV-synthetase physically confined within porous filtration unit.

The repeated use of ACV-synthetase immobilized by physical confinement in an Amicon filtration unit was investigated to determine both efficiency of this system in producing ACV, at 22-24°C and with gentle stirring under a nitrogen or air atmosphere. At the end of each use the reaction mixture was removed by ultrafiltration and the enzyme washed twice with 5 mL of 100 mM Tris/HCl buffer pH 8.5 containing 2 mM DTT. Immobilized ACV-synthetase, maintained under a nitrogen atmosphere, was repeatedly used to produce ACV up to a concentration of 0.25 mM, representing a 25% conversion of the limiting substrate cysteine in 3 h (Table 3.6). The production levels began to decrease after the fourth use, but still attained a production level of 0.16 mM ACV at a much slower rates, after a fifth use. Following several repeated uses, the enzyme retained 45 to 65% of its initial operational activity after 24 h. In the absence of a nitrogen atmosphere the production level was significantly reduced after the fourth use. The immobilized enzyme system appeared unable to produce more than 0.25 to 0.30 mM of ACV with a same reaction mixture, and certain batches of enzyme were unable to sustain linear rates of production for more than 1 h, with ACV production levels below 0.05 mM.

Table 3.6 Effect of repeated use of ACV-synthetase on ACV formation under nitrogen or air atmosphere.

Number of use # (1)	Total time elapsed (3) (h)	Conversion of 1 mM Cys (%)(4)	
		Nitrogen	Air
1	3.0	24.6	25.4
2	6.5	25.9	25.3
3	10.0	22.0	19.0
4(2)	14.0 (20.0)(2)	11.0 (25.9)	4.9 (7.0)
5	23.0	16.0	5.9

(1) Each normal use represents a 3-h reaction period, except

(2) for use # 4, the conversion values in brackets correspond to the values estimated after an additional 6 h period of operation to the normal 3-h use, resulting in a total reaction time of 9 h before reaction mixture was replaced with fresh reagents.

(3) Total time elapsed from first use to the fifth use, including washings.

(4) Conversion is estimated on the basis of the amount of ACV produced (μmol) from the initial amount of cysteine (5 μmol) in 5 mL of the standard reaction mixture.

3.3.3.3 Analysis of factors affecting higher conversion levels.

Analysis of the effects of reaction products indicated no significant effect of ACV on the catalytic rate of ACV-synthetase at concentrations of up to 137 μM. However, due to the nature of the enzyme assay system, higher concentrations of ACV could not be tested since they increased the background level of ACV to levels that did not permit an accurate determination of product formed from catalytic activity alone.

Other reaction products were tested as potential inhibitors of ACV-synthetase. The enzyme activity was inhibited by AMP, PP_i, and ADP, at 5 mM each, by 90, 62 and 40%, respectively. AMP inhibition was strongest, but only at concentrations in excess of those formed in reaction mixtures (Table 3.7). The addition of adenylate kinase, adenylic acid deaminase, or inorganic pyrophosphatase to the reaction mixture did not improve the conversion efficiency, suggesting that AMP and PP_i were not likely the primary cause for preventing the immobilized ACV-synthetase reactor to achieve conversion higher than 30%.

The effect of glycerol, a component of enzyme buffer system, on the catalytic activity of ACV-synthetase was also analyzed. Results showed a reduction in the catalytic rate of the enzyme with increasing glycerol concentrations. However, HPLC analysis of the negative control samples of standard reaction mixtures containing glycerol at concentrations of 30% (v/v) or greater, revealed a peak having the same retention time as ACV, in the absence of the enzyme. This interference was not observed with negative control samples were prepared in water or buffer, suggesting it to be likely a result of a chemical reaction within the reaction mixtures.

To minimize the effect of the components in the standard enzyme storage buffer altogether, a concentrated enzyme preparation was made by resuspending the ammonium sulfate pellet in a smaller volume of 100 mM Tris/HCl buffer, pH 8.5 plus 2 mM DTT. After removal of the salt by gel filtration, this partially purified ACV-synthetase was used to analyze the effect of enzyme amount on the efficiency of the reactor. Typical saturation

Table 3.7 Effect of reaction product AMP and AMP-utilizing enzymes on the yield of ACV by immobilized ACV-synthetase.

AMP conc. (mM)	co-immobilized enzyme(1)	% conversion(2)
0.0	NE	26.9
0.5	NE	26.3
2.0	NE	14.0
5.0	NE	3.8
0.0	AK + AD	22.6
5.0	AK	5.2
5.0	AD	2.4
5.0	AK + AD	5.2

(1) The effect of AMP at indicated concentration was analyzed in the absence of an exogenous enzyme (NE), or in the presence of adenylate kinase (AK) at 320 mU/mL, or adenylic acid deaminase (AD) at 84 mU/mL, or both.

(2) Conversion is estimated on the basis of the amount of ACV produced (μmol) from the initial amount of cysteine (1 μmol) in 1 mL of the standard reaction mixture. The reaction was carried out at room temperature, for 3 h, under a nitrogen atmosphere.

curves of product formed with increasing enzyme amounts were not observed at much higher protein concentrations. That is, rather than the reaction proceeding faster with higher production levels at higher enzyme concentrations, the specific production rates decreased rapidly and the maximum production level also decreased (Table 3.8). The production levels at higher enzyme concentration could not be enhanced by making incremental additions of the limiting and inhibitory substrate cysteine. This suggested that at higher protein concentrations, the reaction products from other contaminants or competing reactions in this enzyme system resulted in the inhibition of ACV-synthetase activity.

3.4 Overexpression, refolding, and characterization of ACV-synthetase domain-1.

The last part of this research project involved the production and characterization of the recombinant ACV-synthetase domain-1. ACV-synthetase contains 3 domains (Aharonowitz *et al.*, 1993), each of which is believed to bind only one of the three substrate amino acids. The DNA fragment encoding the putative first domain of *S. clavuligerus* ACV-synthetase, approximately 3 kb in size, had been cloned on an expression vector backbone from pOW382 to generate the 9.4 kb-plasmid, pOW409 (Figure 3.22, courtesy of Dr. Kovacevic, Eli Lilly). This plasmid contained the tetracycline resistance marker, the domain-1 gene was under the control of the P_L promoter for heat induction, and was introduced in *E. coli* strain JM109 for expression. The objective of these studies was to obtain sufficient amount of the putative domain-1 polypeptide from *E. coli* to enable its characterization.

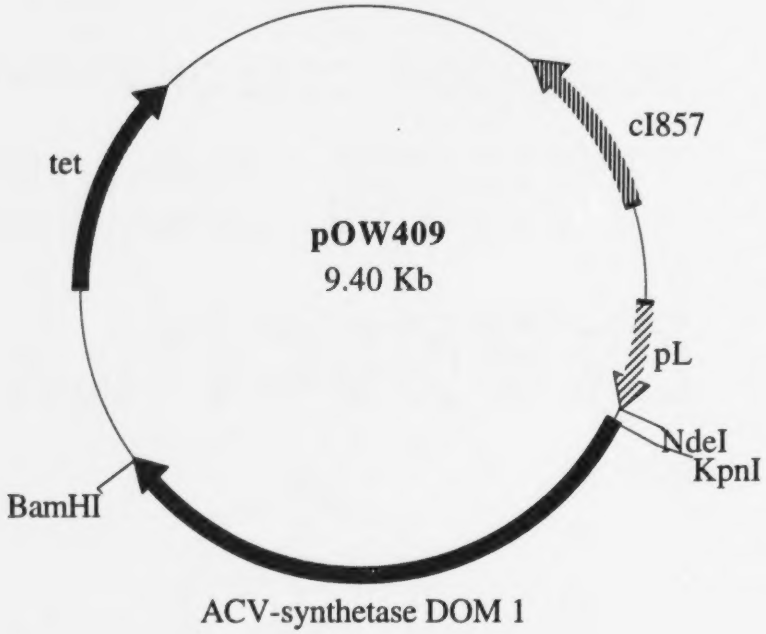
At the onset of this project, some attempts had already been made by others to produce and characterize the domain-1 polypeptide. From these studies, the following conclusions were made: (i) the heat inducible expression system of *E. coli* strain pOW409/JM109 was the best for production of domain-1 polypeptide, (ii) this polypeptide was expressed as inactive inclusion bodies, (iii) there were problems with the solubilization

Table 3.8 **Effect of enzyme amount on the efficiency of the immobilized ACV-synthetase reactor.**

Enzyme amount (1) (mg)	ACV formation (μM) 1 h	ACV formation (μM) 2 h
0.3	24.2	55.8
0.6	41.4	85.8
1.5	69.7	86.4
2.4	53.9	59.6
3.0	41.4	47.4
4.2	28.8	29.4

(1) The amount of partially purified enzyme in 100 μL of standard reaction mixture.

Figure 3.22 Plasmid construct of pOW409 containing the ACV-synthetase domain-1 DNA fragment, (courtesy of Dr Kovacevic, Eli Lilly).



tet
cI857
pOW409
9.40 Kb
pL
NdeI
KpnI
BamHI
ACV-synthetase DOM 1

and renaturation processes, and (iv) no conclusive evidence on the function of domain-1 had been obtained.

These studies were repeated, and similar observations were made with regard to the solubilization, refolding and characterization of this polypeptide. In the following studies attempts were made to optimize these processes.

3.4.1 Improvement of production yield of ACV-synthetase domain-1 polypeptide.

A typical final OD_{600} of the induced *E. coli* pOW409/JM109 culture was around 1.2 in LB medium. To increase the biomass production, different growth media were investigated. Among these were TY (Tryptone, yeast extract), 2x TY, and Stanley Tabor medium. After an overnight growth at 28°C, the Tabor's medium supported the highest growth with a culture OD_{600} of 6.2, whereas the OD_{600} of cultures grown in 2x TY and TY media were 4.6 and 3.1, respectively (Table 3.9). As a result, the Tabor medium (Tabor and Richardson, 1985) was selected as the growth medium for the expression of the domain-1 polypeptide.

Routinely, the culture was grown as a 100-mL culture in a 500-mL flask to an OD_{600} of 1.2-1.5, typically after 3-5 h of growth from a 2% inoculum of an overnight seed culture, at 28°C. The culture was then heat-shocked at 42°C for another 3-4 h before harvesting, at which time the final OD_{600} was about 3.0. Two hours after the heat induction, sparse granules became visible inside the cells and were localized mainly at one end of the cell, and occasionally at both ends or in the center. At this time, the cells were getting smaller, mainly single short rods as opposed to the larger cells from uninduced cultures. Uninduced cultures also contained both paired and single cells. Four hours after induction, the harvested cells contained large inclusions bodies localized primarily at one end of the cell, that were easily detected by phase-contrast microscopy. Figure 3.23A

Table 3.9 Medium composition and its effect on overnight growth of *E. coli* pOW409/JM109.

	Medium(1)		
	TY	2x TY	Tabor (2)
Tryptone (g/L)	10	16	20
Yeast extract (g/L)	5	10	10
NaCl (g/L)	10	5	5
Glycerol (g/L)	0	0	2
Growth (OD_{600}/mL)	3.1	4.6	6.2

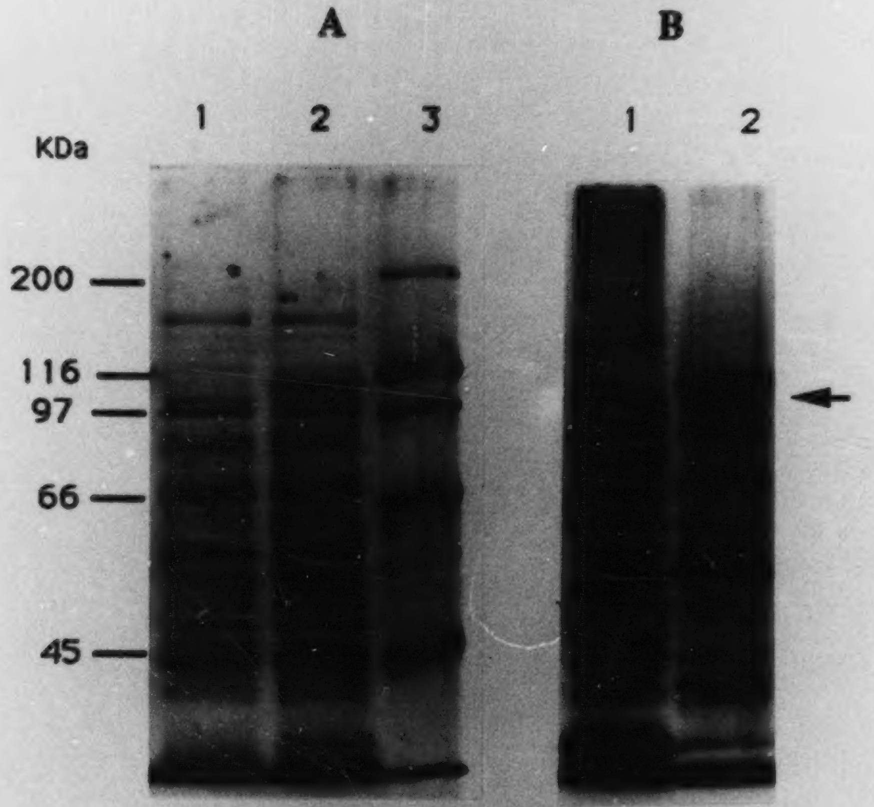
(1) All media contained tetracycline (5 mg/L)

(2) Tabor medium (Tabor and Richardson, 1985) also contained potassium phosphate (50 mM), pH 7.2

Figure 3.23 SDS-PAGE analysis of ACV-synthetase domain-1 expression.

A: 10% polyacrylamide gel of cellular proteins of uninduced (lane **1**) and induced cells of *E. coli* JM109 (lane **2**). Lane **3** consists of protein markers: myosin (200 kDa), ß-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), hen egg ovalbumin (45 kDa).

B: 10% polyacrylamide gel of the soluble cellular proteins (lane **1**) and domain-1 inclusion bodies (lane **2**) from induced *E. coli* cells. Arrow shows the location of ACV-synthetase domain-1. The lanes contain 20-30 μg protein.


A
B
1
2
3
1
2
KDa
200
116
97
66
45

shows the presence of domain-1 polypeptide band with a MW of about 100 kDa, exclusively in the induced cells.

The inclusion bodies were recovered from the cell extracts as a creamy-white pellet by differential centrifugation, after the cells were broken open. Analysis of the protein distribution in various fractions of the sonicated cells showed that the inclusion bodies constituted 21% of the total cellular protein. The efficiency of the sonication procedure was demonstrated by the significant reduction of protein in the soluble fractions after three rounds of sonication, with 67%, 10.7% and 1.3% of total cellular protein recovered after the first, second and third sonication. SDS-PAGE analysis indicated that most of the domain-1 polypeptide was in the inclusion bodies, and constituted the majority of the proteins, as shown by the intensity of the 100-kDa band seen on the gel, as well as some contaminating bands (Fig. 3.23B). The same protein banding profile was observed even when the cell free extract was prepared from sphaeroplasts and the inclusion bodies treated with the detergent Triton X-100, according to the procedure of Lin and Chen (1991) for partial purification of inclusion bodies.

The protein content in inclusion bodies was analyzed by dye-binding and Lowry assays (Bradford, 1976; Lowry *et al.*, 1951). When the inclusion bodies in water were analyzed by the dye-binding method, they constituted only 9% of the total cellular protein . This value was even lower when the inclusion bodies were treated with Triton X-100 to solubilize contaminating membrane proteins. However, the solubilization of the inclusion bodies with 8 M urea or 6 M guanidine hydrochloride, resulted in at least a two-fold increase in the estimated yield of protein. Protein estimation by the Lowry assay, assuming that the NaOH-heat treatment would dissociate the polypeptide units, gave values that were low and similar to dye-binding estimates. Thus the NaOH-heat treatment was ineffective in solubilizing the aggregates.

3.4.2 Inclusion body solubilization studies.

The solubilization of the domain-1 polypeptide inclusion bodies was investigated primarily with urea, but also with guanidine hydrochloride. The effect of the denaturant during the solubilization step was monitored by estimating the amounts of protein in the soluble fraction of the denaturant-treated inclusion bodies. Spectral analyses of aggregated polypeptide and urea-solubilized polypeptide showed characteristic spectral profiles (Fig. 3.24). The urea-denatured soluble polypeptide absorbed only in the U.V. region, with a well defined sharp peak at 280 nm. On the other hand the aggregated polypeptide absorbed in both the UV and visible regions, with a peak at 230 nm which decreased into the visible region, likely due to light scattering of the particulates. It was also observed that an absorption (or turbidity) reading at 320-340 nm gave the largest difference in the readings between the aggregated and denatured polypeptide samples. While measurements at this wavelength were also useful for the analysis of the two states, spectral analysis gave more complete information and was the preferred method in the subsequent studies.

Analysis of various parameters affecting effective solubilization of the domain-1 polypeptide was carried out by resuspending domain-1 inclusion bodies (3 mg protein/mL) in the standard denaturation buffer (100 mM MOPS/KOH, pH 7.5, 50 mM DTT, 5 mM EDTA, 20 % glycerol, and denaturant). Two denaturants, urea or guanidine hydrochloride, were investigated at different concentrations. After incubation at room temperature for a period of 2 h, spectral analysis showed a change in the spectrum of the inclusion body preparation dependent on the denaturant concentration. On the basis of the reduction in turbidity, the effective concentration of urea and guanidine hydrochloride to solubilize the aggregates was estimated to be 8.0 M, and 6.0 M, respectively (Fig. 3.25). This correlated well with the observations made by SDS-PAGE, which also showed increases in the amounts of proteins in the soluble fractions of urea-treated inclusion bodies (Fig. 3.26). The guanidine hydrochloride-treated samples could not be analyzed by SDS-PAGE, as the

Figure 3.24 Spectral analysis of soluble and aggregated forms of ACV-synthetase domain-1 polypeptide.

Inclusion bodies of ACV-synthetase domain-1 were resuspended in 8 M-urea MOPS-based denaturation buffer at a protein concentration of 2.7 mg/mL (---), and in the same buffer without urea at a protein concentration of 0.27 mg/mL (—), and the absorption spectrum of the samples was analyzed. The absorption contribution in the UV region due to DTT present in the buffer was subtracted from the profiles.

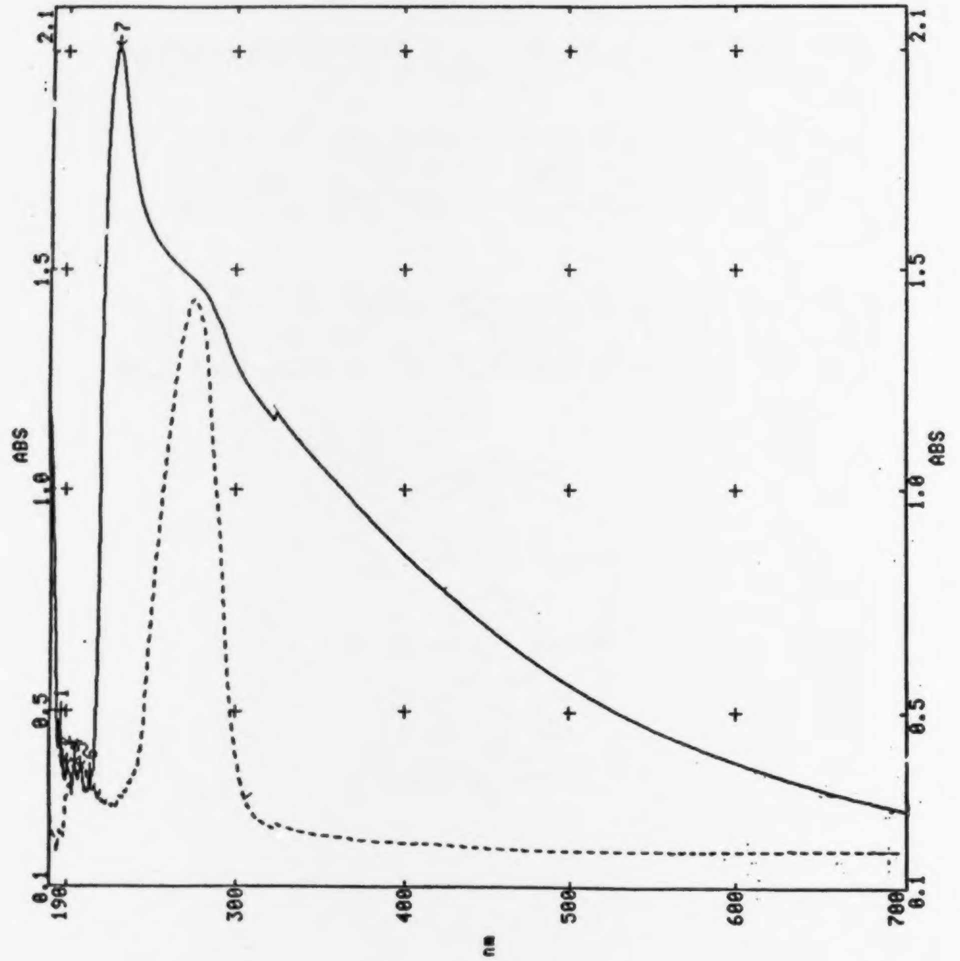

ABS
2.1
1.5
1.0
0.5
0.1
nm
190
300
400
500
600
700
7
1

Figure 3.25 Spectral analyses of the effect of denaturant concentration on the solubilization of domain-1 polypeptide inclusion bodies

Inclusion bodies of ACV-synthetase domain-1 were resuspended in the MOPS-based denaturation buffer at a protein concentration of 2.7 mg/mL in the presence of the denaturant at concentrations of 0.8, 2.0, 3.5, 5.0, 6.5 and 8.0 M for urea (**A**); and 0.6, 1.5, 3.0, 4.5 and 6.0 M for guanidine hydrochloride (**B**), and the absorption spectrum of the samples was analyzed. The absorption contribution in the UV region due to DTT present in the buffer was not subtracted from the profiles.

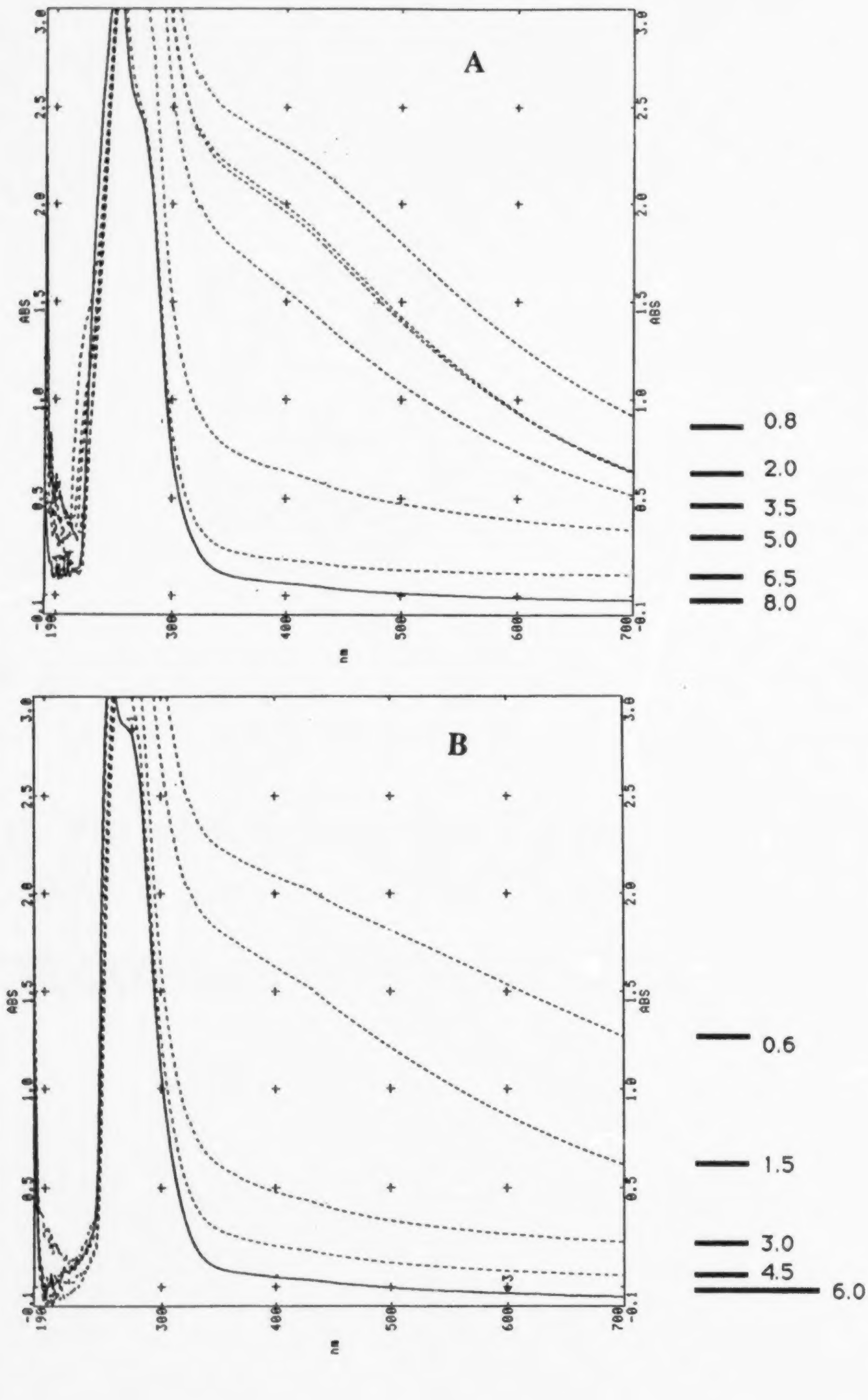
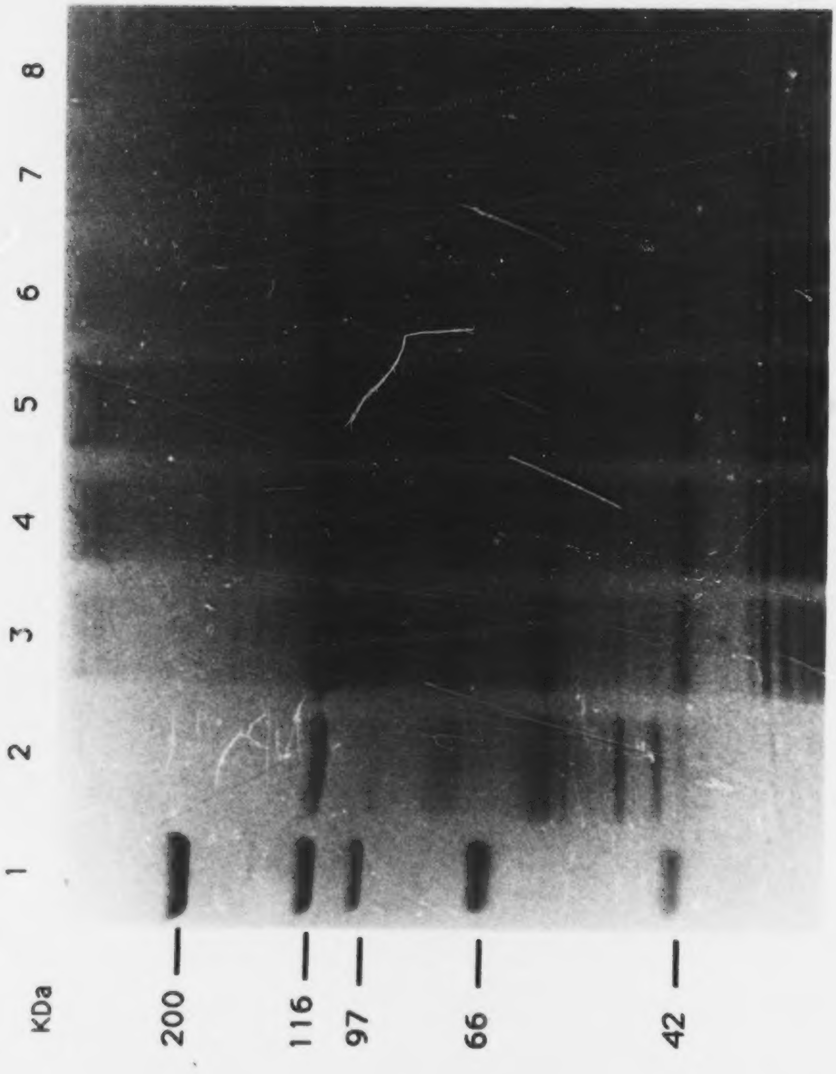

A
ABS
3.0
2.5
2.0
1.5
1.0
0.5
-0.1
190
300
400
500
600
700
nm
0.8
2.0
3.5
5.0
6.5
8.0
B
0.6
1.5
3.0
4.5
6.0

Figure 3.26 SDS-PAGE analysis of the urea concentration effect on the solubilization of domain-1 polypeptide inclusion bodies.

Inclusion bodies of ACV-synthetase domain-1 contained in a storage buffer were recovered by centrifugation, and washed twice with buffer with a centrifugation step in between. The first supernatant was saved and a sample of this loaded in Lane **2**. The inclusion bodies were resuspended in the MOPS-based denaturation buffer at a protein concentration of 2.7 mg/mL in the absence or presence of the urea at different concentrations. After a 2 h incubation at room temperature the urea treated samples were microfuged for 10 min, and 15 μL of the supernatant analyzed by SDS-PAGE, 7.5% gel. Samples consist of standard proteins (Lane **1**), urea untreated inclusion bodies not centrifuged (Lane **3**), soluble fractions of urea-treated samples: 8 M (Lane **4**), 6 M (Lane **5**), 2 M (Lane **6**), 0.8 M (Lane **7**), and soluble fraction of urea-untreated inclusion bodies (Lane **8**).


1
2
3
4
5
6
7
8
KDa
200
116
97
66
42

guanidine hydrochloride-polypeptide complex formed a precipitate with SDS at room temperature. Therefore 8 M urea was selected for subsequent denaturation studies.

3.4.3 Renaturation of the domain-1 polypeptide studies.

Numerous approaches have been used to maximize the recovery of properly folded polypeptide from inclusion bodies (Cleland *et al.*, 1992; Creighton, 1986; Mitraki and King, 1989), and several of these methods were investigated with domain-1 polypeptide.

3.4.3.1 Co-solvent-assisted refolding by dialysis and quick dilution.

Earlier studies had used gel filtration to remove urea from denatured aggregated domain-1 polypeptide but the product was inactive. The removal of urea by dialysis or quick dilution in the presence or absence of a co-solvent was investigated as an alternative mechanism for renaturation . The renaturation was attempted by dialyzing away overnight at 4°C, or diluting out the 8 M urea from denatured aggregated polypeptide under a variety of conditions. Dialysis against the standard renaturation buffer (100 mM MOPS/KOH, pH 7.5, 2 mM DTT, 5 mM EDTA, 20 % glycerol) alone or supplemented with intermediate urea concentrations (2 M and 4 M), or supplemented with PEG (0, 10, 20, and 30 g/L), resulted in protein reaggregation: a milky opalescence, as opposed to the clear urea-denatured protein sample. To determine if the observed reaggregation was favored by the high protein concentration (3 mg/mL) used in these studies, the experiment was then repeated at lower protein concentrations (0.3 and 0.06 mg/mL), against urea-free buffer plus 10 g/L PEG. After dialysis, the samples showed no visible aggregation, but concentration of these samples by ultrafiltration revealed aggregates. Alternatively, the denatured samples (3 mg protein/mL buffer) were quickly diluted in the renaturation buffer to much lower protein concentrations, which also resulted in a dilution of urea, carried out either in the presence or absence of PEG. After ultrafiltration of the samples in the presence or absence of PEG, the results were again similar to those reported above with higher protein concentrations. It was concluded that these approaches were not suitable for the renaturation of domain-1 polypeptide.

3.4.3.2 Refolding by ion-exchange and gel filtration chromatography.

The folding of proteins adsorbed reversibly to ion-exchange resins has been demonstrated (Creighton, 1986), and this procedure was investigated with the domain-1 polypeptide. Initial renaturation studies were carried out on a Mono Q column by FPLC. The protein (6 mg) was denatured in 2 mL of the standard denaturation buffer containing urea at a concentration of 8 M. At the end of the solubilization reaction (2 h), the domain-1 polypeptide solution was diluted with an equal volume of the standard renaturation buffer supplemented with PEG (10 g/L). This 4 M urea sample was loaded to a column pre-equilibrated in a low ionic strength renaturation buffer A (50 mM MOPS/KOH, pH 7.5, 2 mM DTT, 20 % glycerol), containing PEG (5 g/L), and then eluted with an increasing linear gradient of a high ionic strength renaturation buffer B (500 mM MOPS/KOH, pH 7.5, 2 mM DTT, 20 % glycerol). Two peaks were detected in the effluent fractions but only small amounts of protein were recovered. The protein composition of these two peaks was similar to non-urea treated samples. Several variations on this procedure were carried out but only minor improvements were achieved. Apparently much of the domain-1 polypeptide was binding tenaciously to the column. Thus, to avoid damaging the Mono Q column with unrecoverable aggregated polypeptide, this procedure was discontinued.

Attempts were made to slowly change the refolding conditions using the ion-exchange column DEAE-Trisacryl. The strategy involved the combination of the quick-dilution procedure in the presence of a co-solvent (PEG). In two separate experiments, 8-M-urea denatured polypeptide (6 mg in 2 mL of the standard denaturation buffer) was quickly diluted 10-fold in renaturation buffer supplemented with 4 M urea and 1 g/L PEG, or with 2 M urea and 30 g/L PEG in a second study. After incubation at 4°C for 1 h, samples were loaded onto DEAE-Trisacryl columns, and the urea and PEG eluted with two separate linear gradients, before elution of the protein with high salt. As with the Mono Q study, most of the protein remained on the column.

Several other ion-exchange matrices were studied, including CM-52 cellulose, CM-Trisacryl and DEAE-Sephacel, using a solution of urea denatured domain-1 polypeptide in a batch system and eluting with high salt. Whether in batch mode or eluted from a column, only low levels of protein were recovered. These results implied the involvement of other types of interaction between the resins and the solubilized protein, presumably hydrophobic in nature.

An alternative method of ion-exchange chromatography was a batch system, where the aggregated protein is reacted with the resin with no prior solubilization of the inclusion bodies (Hoess *et al.*, 1988). Two ion-exchange matrices: DEAE-Trisacryl and aminopropyl CPG, earlier shown to bind ACV-synthetase, were investigated. Using the published methodology, 60-80% of the particulate polypeptide material was bound to the matrix but only polypeptide of particulate nature was released by the eluant, based on the spectral analysis of the samples.

Gel exclusion chromatography was also used to purify the domain-1 polypeptide from contaminating proteins present in inclusion bodies. Results using desalting columns suggested that the product recovered was mainly composed of re-aggregated polypeptide. When columns of Sephadex G-100 and G-200 were used, in both cases the domain-1 polypeptide was recovered in the exclusion volume. SDS-polyacrylamide gel profile showed that no purification of the domain-1 polypeptide from other lower molecular weight proteins was achieved, suggesting co-migration of the inclusion body proteins as aggregates. Although London *et al.* (1974) demonstrated that the degree of purity of *E. coli* tryptophanase did not affect the aggregation of this enzyme during renaturation, attempts were still made to purify ACV-synthetase domain-1 by Sephadex G-200 chromatography under denaturing conditions (7.2 M urea), and then analyze its refolding in the absence of contaminants, but most of the domain-1 polypeptide, including contaminating proteins, was eluted in the void volume.

3.4.4 Further optimization of the solubilization and renaturation conditions for ACV-synthetase domain-1 polypeptide.

The inability to purify the unfolded domain-1 polypeptide by gel filtration suggested that the solubilization conditions were not optimum, despite evidence of solubilization on the basis of spectral analysis and SDS-PAGE analysis. Therefore, the buffer system and the urea concentration during solubilization, and the pH during renaturation were analyzed. The effect of pH in the range of 5 to 9 on the renaturation of unfolded domain-1 polypeptide was studied. It was found that the milky appearance associated with the reaggregation of the protein sample decreased with increasing pH. This was confirmed from spectral analysis, suggesting that reaggregation was minimized at pH 8.5-9.0. Thus renaturation was subsequently carried out in Tris/HCl buffer, pH 8.5, instead of MOPS/KOH buffer, pH 7.5. Reducing the glycerol concentration from 20% to 5% and increasing the urea concentration from 8 to 9 M prevented the reaggregation of polypeptide during an overnight refolding reaction, at 10°C. These changes also prevented the milky appearance of the polypeptide renatured either by quick dilution or dialysis at low protein concentrations. This suggested that the reaggregation observed throughout these studies was partly due to incomplete solubilization of the inclusion bodies in 8 M urea, and inefficient refolding in the MOPS/KOH buffer at pH 7.5.

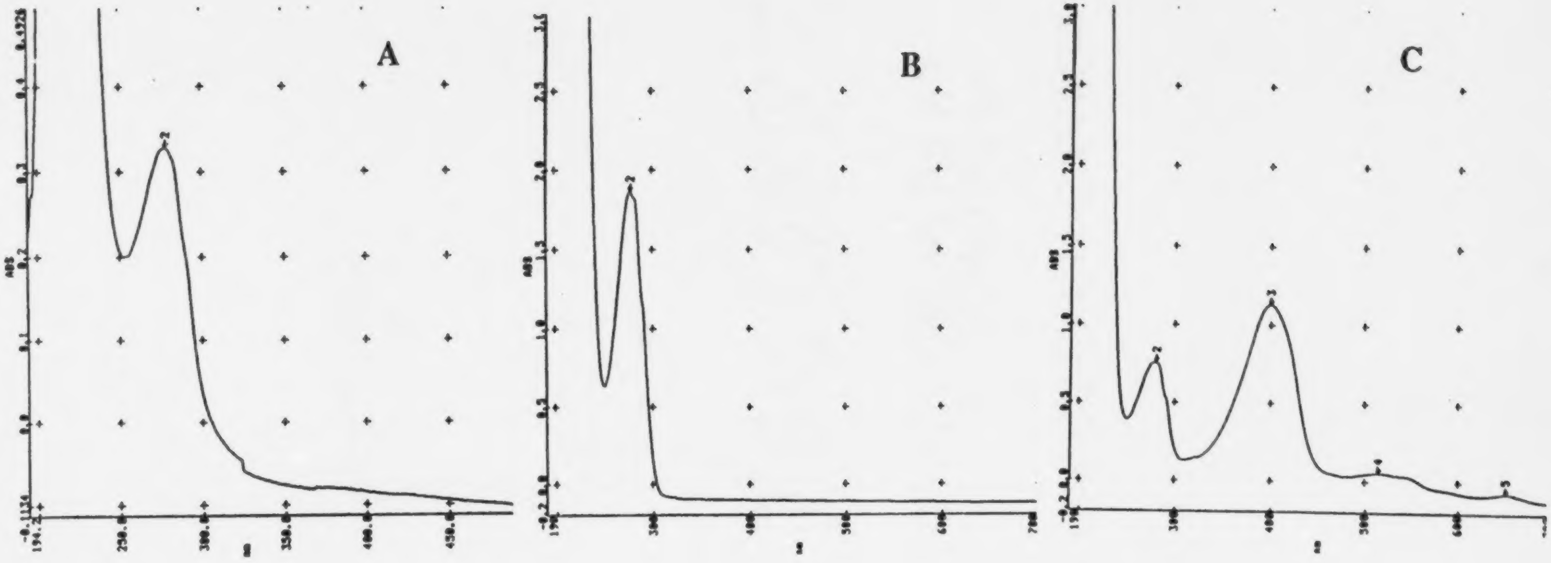
Spectral analysis of the renatured domain-1 polypeptide following the modified procedure showed an absorption peak at 280 nm, typical of soluble protein solutions such BSA or chloroperoxidase (Fig. 3.27). The renatured domain-1 polypeptide was analyzed by SDS-PAGE, and the domain-1 band was visible as a polypeptide of about 100 kDa. However, under non-denaturing conditions the sample was unable to enter the gel, regardless of whether the renaturation was carried out in the presence of PEG or

Figure 3.27 Spectral analysis of refolded ACV-synthetase domain-1

A: Refolded domain-1 polypeptide was prepared in the presence of 0.05% SDS and the absorption spectrum of the sample was analyzed.

B: Spectrum of native soluble bovine gamma globulin.

C: Spectrum of native soluble chloroperoxidase.


A
B
C
ABS
nm
-0.1126
0.0
0.1
0.2
0.3
0.4
0.4926
194.2
250.0
300.0
350.0
400.0
450.0
-0.2
0.0
0.5
1.0
1.5
2.0
2.5
3.0
190
300
400
500
600
700
2
3
4
5

dimethylsulfoxide (0.1% each), and the renatured domain-1 polypeptide solution had remained clear after concentration by ultrafiltration. The possibility could not be ruled out that under these conditions, the refolded polypeptide molecules were still capable of re-associating with each other. Thus the masking of the "sticky site", the hydrophobic amino acids at the junction of this domain to others of ACV-synthetase, was therefore necessary to prevent re-association.

SDS is an amphipathic detergent which at low concentration can react with hydrophobic surfaces on certain native protein without denaturing the protein (Cooper, 1976). The electrophoresis of the renatured domain-1 polypeptide sample was repeated this time under "pseudo non-denaturing" conditions in the presence of 0.05% SDS, without treating the samples as done under denaturing conditions. It was found that the domain-1 polypeptide migrated slightly slower than it normally does under denaturing conditions (Fig. 3.28A). To confirm that the observed effect was not a result of the presence of SDS in solution, the SDS-treated domain-1 polypeptide was washed with buffer by ultrafiltration and then resuspended in water. The absorption spectrum of this water resuspended domain-1 polypeptide was found to be unchanged; and the sample run in normal non-denaturing PAGE showed the domain-1 polypeptide band (Fig. 3.28B). It was also observed that the ACV-synthetase inclusion bodies could also be solubilized with a 0.05% SDS solution. These observations suggest that SDS has likely masked the hydrophobic reactive surfaces on the refolded domain-1 polypeptide.

3.4.5 Characterization of the refolded ACV-synthetase domain-1 polypeptide.

To determine the function of the putative domain-1 of ACV-synthetase, three ^{14}C-labelled amino acids were used with domain-1 polypeptide renatured under five different conditions. These included the renaturation buffer only, or the renaturation buffer supplemented with NaCl (0.5 M), PEG (2%), Triton X-100 (1%) and SDS (0.05%). The

Figure 3.28 SDS-PAGE analysis of domain-1 polypeptide refolded by quick dilution under a variety of conditions.

A: 7.5% polyacrylamide gel under denaturing conditions (Laemmli, 1970).

B: 7.5% polyacrylamide gel under "pseudo-non-denaturing": 0.05% SDS and samples not heat treated.

1. Urea-untreated inclusion bodies (control, 90 µg)

2. Supernatant from (**1**), obtained as described in the legend to Figure 3.26

3. Inclusion bodies (2.4 mg protein) denatured with 9 M urea in 0.4 mL MOPS-based denaturation buffer containing 2.5% glycerol and 50 mM DTT, and incubated for 2 h, at room temperature. A portion of the solubilized polypeptide solution (0.1 mL, 0.6 mg) was quickly diluted 30-fold in 100 mM Tris-based renaturation buffer, pH 8.5. After an overnight incubation at 10°C, the dilute domain-1 polypeptide solution (3 mL) was concentrated by ultrafiltration to 0.3 mL, and 20-25 µg protein loaded.

4. As in (**3**) but diluted into buffer containing 0.1% dimethylsulfoxide

5. As in (**3**) but diluted into buffer containing 0.1% PEG

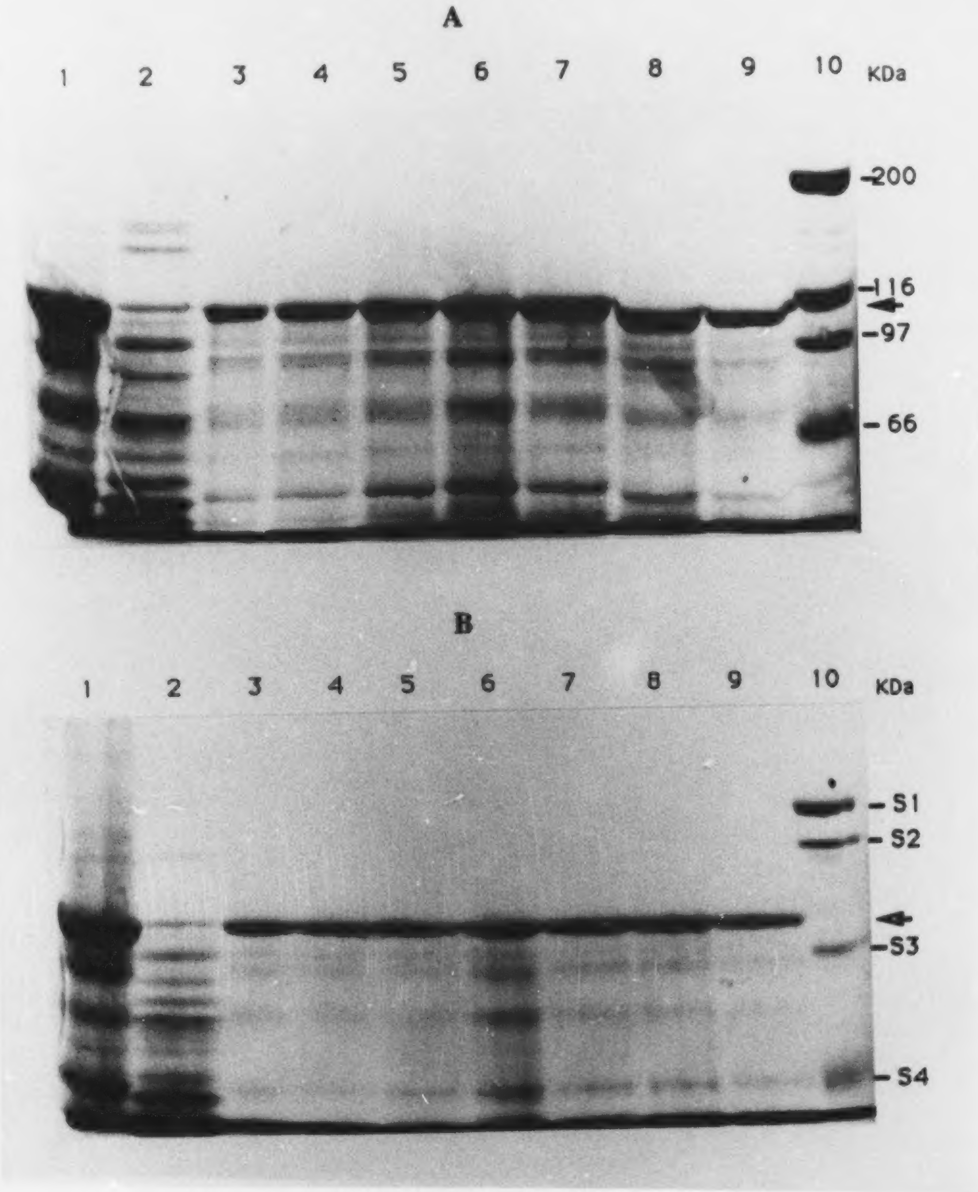
6. As in (**3**) but diluted into buffer containing 0.05% SDS

7. As in (**3**) but diluted into buffer containing 0.05% PEG

8. As in (**3**) but diluted into buffer containing 1% PEG

9. As in (**3**) but diluted into buffer containing 3% PEG

10.Molecular weight markers 200, 116, 97, 66 and 45 kDa, are myosin, ß-galactosidase, phosphorylase b, bovine serum albumin, and hen egg ovalbumin, respectively. ACV-synthetase domain-1 protein band is indicated with an arrow. Due to the change in the migration profile in Panel B of the individual standards under non-denaturing condition, and their localization under these conditions not completely determined, the 4 standard bands are labelled as S1 to S4.


A
1
2
3
4
5
6
7
8
9
10
KDa
-200
-116
-97
- 66
B
1
2
3
4
5
6
7
8
9
10
KDa
- S1
- S2
-S3
- S4

amino acid binding activity of this apparently refolded polypeptide was investigated with ^{14}C-labelled amino acids: L-α-aminoadipate, L-cysteine and L-valine. A specific binding with only ^{14}C-L-α-aminoadipate was observed with the polypeptide renatured under all the conditions tested, except with Triton X-100 (Table 3.10).

Table 3.10 ^{14}C-Amino acid binding activity of ACV-synthetase domain-1 peptide refolded under a variety of conditions.

	cpm(2)		
Refolding condition(1)	L-AAA	L-cysteine	L-valine
Buffer	1908.8	34.6	0.6
Buffer + 0.5 M NaCl	1972.0	16.0	0.0
Buffer + 2% PEG	2032.0	3.7	3.7
Buffer + 0.05% SDS	2014.1	39.8	33.6
Buffer + 1% Triton	166.4	49.35	2.6

(1) Domain-1 polypeptide inclusion bodies (3.0 mg protein) were solubilized in 9 M urea (4 mL) for 2 h at room temperature, then clarified at low speed for 10 min. The solubilized protein was diluted 30-fold (600 µL to 18 mL) in the renaturation buffer (100 mM Tris/HCl, pH 8.5, 10% glycerol, 5 mM DTT) alone or supplemented with NaCl (0.5 M), PEG (2%), SDS (0.05%), and Triton X-100 (1%). The refolding reaction was carried out overnight at 10°C. The protein samples were then concentrated by ultrafiltration to 600 µL, and small aliquots of this (150 µL) used in the ^{14}C-amino acid binding assay mixture (200 µL total volume).

(2) cpm: count/min.

4. DISCUSSION.

4.1 Analysis, production and stabilization of ACV-synthetase from *S. clavuligerus.*

The variations in the estimation of ACV-synthetase activity with ATP concentrations in the assay mixtures and the degree of enzyme purity point out some of the difficulties that have affected the development of a reliable assay for crude and purified enzyme. Since the original conditions for a cell-free ACV synthesis system were defined by Banko *et al.* (1987), ATP concentrations in assay mixtures have varied considerably : with 1 mM (Jensen *et al.*, 1988), 5 mM (van Liempt *et al.*, 1989), 10 mM (Zhang *et al.*, 1987, 1989), and 15 mM (Banko *et al.*, 1986, 1987; Zhang *et al.*, 1987), all in an attempt to optimize the measurement of ACV production.

The main modifications introduced by Jensen *et al.* (1988) to the original assay (Banko *et al.*, 1986, 1987) were a reduction of ATP concentration from 10 to 1 mM, and the inclusion of an ATP-regenerating system consisting of pyruvate kinase and PEP. This assay was developed for use with the crude enzyme, prior to the purification of ACV-synthetase, and showed an improvement in crude enzyme activity. The reevaluation of this assay with purified enzyme in these studies revealed that high ATP concentrations were inhibitory to both crude and purified enzyme, but the stimulatory effect of the modified conditions on crude enzyme was not observed with purified enzyme. The stimulation was due to the supplementation of PEP to the reaction mixtures and required unknown factors present in crude cell-free extracts, but not exogenous pyruvate kinase activity. Pyruvate kinase plus PEP were unable to stimulate the activity of purified ACV-synthetase at low ATP concentrations. This is evident from the analysis of the reactions catalyzed by ACV-synthetase and and pyruvate kinase. As discussed latter with labelled ATP studies, ACV-synthetase reaction generates AMP and PP_i from ATP, and pyruvate kinase generates ATP and pyruvate from PEP and ADP (Worthington, 1988). Without ADP formation, as is the case in the reaction of ATP and AMP catalyzed by adenylate kinase (Chiga and Plant, 1960; Noda, 1973), this regenerating system is uncoupled.

In an attempt to characterize the nature of the PEP stimulatory effect, an endogenous PEP-dependent ATP regenerating system, such as the pyruvate kinase and adenylate kinase coupling system was postulated. The effects of potential inhibitors of pyruvate kinase activity, Ca^{+2} (Betts *et al.*, 1968; Mildvan and Cohn, 1967), and the reaction product pyruvate on the activity of partially purified and purified ACV-synthetase were analyzed. This was done to see whether the inhibition of the regenerating system would result in the reduction of ACV-synthetase activity. Only Ca^{+2} was found to be inhibitory, but its effect was strongest with the partially purified enzyme. Because of the complexity of the partially purified enzyme system, no conclusion could be drawn from these studies regarding the nature of the regenerating system involved. The inhibitory effect of Ca^{+2} may partly be due to a competition between Ca^{+2} and Mg^{+2} for ATP.

From this study, the optimized reaction conditions system for assaying ACV-synthetase, regardless of the purity of the enzyme, consisted of 2 to 5 mM ATP and 5 mM PEP. The importance of PEP as a component of the ACV-synthetase assay system was nicely demonstrated during the kinetic analysis of ACV formation with partially purified enzyme, in which the PEP-containing system sustained a linear reaction rate for a longer period of time and at a larger amount of ACV-synthetase protein, and gave higher estimates of ACV-synthetase activity. Recent studies with purified enzyme from *S. clavuligerus* have shown that the optimum reaction conditions were 2 mM ATP, and 10 mM Mg^{+2}, at the reaction temperature of 29 - 30°C, and at the reaction pH of 8.5 in MOPS buffer (Zhang *et al.*, 1992).

An assay system that varies with the purity level of the enzyme can complicate the estimation of enzyme recovery during a purification scheme, due to an underestimation of the specific activity in crude enzyme preparations. Schwecke *et al.* (1992) reported on the purification of ACV-synthetase from *S. clavuligerus*. Their results revealed striking evidence of the difficulty in determining the activity of ACV-synthetase in crude enzyme preparations. The recovery was found to oscillate between 13 and 244% of the initial crude activity in the first three purification steps, based on the incorporation of ^{14}C-valine into ACV at an ATP concentration of 5 mM. Under such conditions competition for ATP in crude cell-free extracts, coupled with a low concentration of valine, would emphasize the underestimation of total activity in crude preparations. Similar observations could be made for the purification table reported by van Liempt *et al.* (1989).

ACV-synthetase, like other peptide synthetases, is very labile (Banko *et al.*, 1986; van Liempt *et al.*, 1989; Zhang and Demain, 1990a, 1992b). Although some biochemical characteristics and immobilization of the enzyme from *S. clavuligerus* have been studied in our laboratory (Jensen *et al.*, 1988, 1989), most of its stability properties have not been systematically characterized. Partial stabilization of the enzyme from *Cephalosporium acremonium* with glycerol by Banko *et al.* (1986) represented an important step for subsequent studies on this enzyme from both fungal and bacterial sources. Since this project was intended to develop an enzymic process for the production of ACV, a systematic analysis of the culture harvest time and stabilization conditions was therefore necessary to optimize both yield and stability of the enzyme.

The dynamics of cephamycin C fermentation by *S. clavuligerus* revealed two distinct phases. The first involved the channelling of the amino acids towards the formation of ACV, and its intracellular accumulation. The second phase corresponded to the period in which this ACV pool was drawn upon for the production of antibiotic which accumulated in the culture fluid. ACV-synthetase was found in the mycelia during both phases, even in older cultures when the rate of total antibiotic formation had become negligible. Yet, from the viewpoint of cellular economy, ACV-synthetase should represent an ideal major target for nutritional control of classical ß-lactam biosynthesis (Piret *et al.*, 1990). In both fungi and bacteria, the appearance of this enzyme should indicate the onset of antibiotic biosynthesis, and its disappearance indicate the curtailment of antibiotic production. In addition, the discovery of the involvement of lysine-ε-aminotransferase in the biosynthetic pathway of classical ß-lactams in bacteria, would further suggest the possible existence of a coordinated regulation of both lysine-ε-aminotransferase and ACV-synthetase as part of the overall control system (Tobin *et al.*, 1991). In this study however, evidence suggested that after the onset of antibiotic biosynthesis, the activity of ACV-synthetase was primarily regulated at the enzyme level, since the enzyme was physically present throughout the course of the fermentation regardless of variations in the rate of total antibiotic production.

Calculation of the *in vivo* activity of ACV-synthetase may not be made easily from *in vitro* enzyme activity studies. This is because inhibitors such as phosphorylated glycolysis intermediates (Zhang and Demain, 1991), the availability and concentration of substrate, and the fact that the enzyme is removed from the presence of other pathway enzymes could affect the rate of product formation *in vitro* (Namdev *et al.*, 1992). This view is supported by the increased sensitivity of crude ACV-synthetase to a variety of effectors, such as PEP and calcium, both of which had no significant stimulatory or inhibitory effect on the purified enzyme. The stimulatory effect of PEP on crude ACV-synthetase activity was presumably mediated by the activation of a PEP-dependent ATP-regenerating system. It could therefore be speculated that the activity of ACV-synthetase *in vivo* may be regulated by ATP availability. The inhibition of ACV-synthetase by easily utilized carbon sources has been reported to be due to competition for ATP in crude cell-free extracts (Zhang and Demain, 1992a). Other studies have also reported on the stimulatory effect by an ATP-regenerating system on crude enzyme activity. For instance, the penicillin N ring expansion process by DAOC synthase was stimulated by ATP with crude but not highly purified enzyme systems (Scheidegger *et al.*, 1984). Yoshida *et al.* (1978) found also that in the presence of the ferrous ions and ascorbate, which are known to stimulate α-ketoglutarate-linked dioxygenases (Shen *et al.*, 1984), the ATP-regenerating system became unnecessary, and the ATP concentration could be significantly reduced with the activity increasing by nearly 60-fold.

Substrate and ATP limitations, brought about by a drop in the intracellular levels of these compounds or an increase in competing reactions, may account for the apparent decline in ACV-synthetase activity in cell-free extracts from older cultures. Changes in intracellular nucleotide pool sizes during growth of *Streptomyces* sp. MA406-A-1, a producer of the nucleoside antibiotic formycin, have been studied by Ochi (1986). It was found that the ATP level drastically increased towards the end of exponential growth in a synthetic medium, coinciding with the beginning of antibiotic production, then dropped soon after in the stationary phase. Time-course studies on the production of cephalosporins by *S. clavuligerus* have also shown dramatic changes in ATP pool size, with a rapid increase during early growth followed by a decline as the level of cephalosporins increased (Bascarán *et al.* 1991). In these studies, the Michaelis constant of ACV-synthetase for ATP was quite high, implying that *in vivo* this enzyme would be more sensitive to variations in ATP levels than some of the other cellular ATP-utilizing enzymes, such as kinases and transphosphorylases (Chiga and Plant, 1960). The results also suggested that the increase in the competition for ATP by cellular ATP-utilizing enzymes may be one of the mechanisms of regulating the activity of this enzyme during the idiophase, possibly in combination with substrate amino acid limitations. These observations reinforce the importance of an ATP regenerating activity in regulating antibiotic production at the enzyme level.

The activity profile of ACV-synthetase was not transient, as has been observed for other non-ribosomal peptide synthetases such as gramicidin S synthetase (Vandamme *et al.*, 1982), bacitracin synthetase (Kleinkauf and von Döhren, 1983), and enniatin synthetase (Billich and Zocher, 1988). In previous reports of ACV-synthetase activity profiles (Zhang *et al.*, 1989a,b), the enzyme activity decayed during the antibiotic production phase. However, as shown from this study, the apparent decay in ACV-synthetase activity during this phase may be due to an effect related to ATP availability, which could be overcome with the addition of PEP. On this basis, the kinetics of production of ACV-synthetase from *S. clavuligerus* are similar to those of the same enzyme from *Penicillium chrysogenum* (López-Nieto *et al.*, 1985). The fermentation of *P. chrysogenum* showed no decay in ACV-synthetase activity when measured *in vivo* by blocking protein synthesis at different times and determining the rate of incorporation of labelled substrate amino acid into ACV by preexisting ACV-synthetase. The *P. chrysogenum* enzyme reached a maximum activity at 48 h, and remained constant for up to 120 h of fermentation.

These studies raise some questions about the cellular function of ACV-synthetase. Microorganisms are most likely to remove proteins that are no longer useful. Since ACV-synthetase remains active longer than needed for antibiotic production, it could be speculated that this enzyme may be playing another role in the cell. Unlike the *Bacillus* non-ribosomal peptide synthetases which direct primary metabolites to an active product, ACV-synthetase produces an intermediate in antibiotic synthesis, not a product. There have been reports of excretion of ACV into the culture fluid of *P. chrysogenum* (Adriaens *et al.*, 1975; López-Nieto *et al.*, 1985), suggesting that in this organism activity is not tightly controlled. Similarly, the ACV precursor L-α-aminoadipate has been reported to be synthesized in excess of the need for incorporation into antibiotic. Madduri *et al.* (1991a) observed the accumulation of extracellular L-α-aminoadipate near the end of the exponential growth, a time at which lysine-ε-aminotransferase activity had declined and the intracellular pool of this amino acid had also declined. This was likely due to the slow conversion of L-α-aminoadipate into ACV. A comparison of several biosynthetic enzymes in crude cell-free extracts of *C. acremonium* and *S. clavuligerus* has indicated that ACV-synthetase has the lowest specific activity of the other three enzymes immediately downstream the pathway (Zhang and Demain, 1991). Does continued L-α-aminoadipate production justify continued production of ACV-synthetase or, alternatively does the continued presence of the substrate enhance the stability of ACV-synthetase *in vivo*? Recent work by Aharonowitz *et al.*(1993a) suggests that ACV and a disulfide reductase may, in the absence of glutathione, be responsible in part for the thiol-disulfide redox balance in *S. clavuligerus*. Such a function could explain the persistence of ACV-synthetase in the cell beyond the end of the antibiotic production phase.

Although ACV-synthetase was present at most culture ages, the enzyme isolated during the period when ACV was accumulating in the mycelia, was the most stable during storage at 4°C. No direct evidence of enzyme inactivation by proteinase was observed, though an intracellular proteinase with a pH optimum at 8.2 and active on azocasein was detected at all culture ages. However, this does not exclude the possibility that other proteinolytic activities, not detectable with azocasein, may be involved in the inactivation of ACV-synthetase *in vitro*. The importance of the physiological state of the cells was also observed by Freeman and Aharonowitz (1981) while analyzing the capacity of *S. clavuligerus* resting cells with that of the free growing cells to produce cephalosporins as a function of culture age. It was found that resting cells prepared from the early log phase, where antibiotic could not be detected in the fermentation broth, were highly active as antibiotic producers, both as soluble and immobilized biocatalysts.

The enhanced stability of the enzyme from mycelia harvested before the appearance (8-h cultures) and the decrease in the intracellular ACV level (38-h or older cultures) could be due to stabilization by low molecular weight molecules, such as substrates. Tasker and Agathos (1989) demonstrated the dependence of the inactivation kinetics of gramicidin S synthetase from *B. brevis* and the degree of its amino acid-mediated stabilization *in vivo* on the growth stage of the culture. The effect may also be simply due to an intrinsic property of the enzyme, so that after ACV has disappeared from the mycelia, the enzyme is marked in some way to become more susceptible to inactivation *in vitro*. Alternatively, limited proteinolysis may be involved in the inactivation of the enzyme from idiophase (Kleinkauf and Koischwitz, 1974, 1980; Vandamme, 1980). This possibility could not be ruled out, since the cell-free extracts were not analyzed for any degradation of ACV-synthetase protein after storage. Zhang and Demain (1990a) have reported on the improvement of ACV-synthetase stability, from a narrower range ammonium sulfate fractionation of the extract, in combination with the incorporation of some additives to the enzyme preparation, suggesting that some factors present in the crude extracts may contribute to enzyme instability.

Evidence of the important role of reducing agents and substrates in the stabilization of the enzyme was confirmed in these studies. Dissolved oxygen was likely an important factor in the observed instability of the ACV-synthetase during storage at 4°C. DTT and glycerol provided the greatest stabilization of the enzyme and the addition of $MgCl_2$ plus the three substrate amino-acids enhanced the stability of partially purified ACV-synthetase. From an independent study (Zhang and Demain, 1992b; Zhang *et al.*, 1992) these additives were also found to improve the stability of the purified enzyme, and among the three substrate amino acids tested, valine was the only substrate found to enhance the enzyme stability *in vitro*. The enzyme half-life was dramatically increased to almost 15 days, at 4°C. These results emphasize the importance of substrate protection of the enzyme, presumably through the protection of the active site or by forcing the enzyme to remain in the native conformation. Maintaining a reduced environment appeared to be an essential requirement for stability of ACV-synthetase. Improvement of the stability of the enzyme by substrates and DTT provides a convenient means of achieving good operational stability of a partially purified ACV-synthetase, for use in studies on the production of ACV.

4.2 Analysis of effect of arginine, glutamate and ornithine on ß-lactam production by *S. clavuligerus*

ACVSR is a 32-kDa protein that co-purified with ACV-synthetase (Jensen *et al.*, 1990). This protein was encoded by a gene that physically mapped in the proximity of the cephamycin biosynthetic gene cluster in *S. clavuligerus* (Aidoo, K. and S.E. Jensen, Personal communication), which extends from *cef*D/*cef*E genes coding for DAOC synthase and isopenicillin N epimerase to *pcb*C gene encoding IPNS (Kovacevic *et al.*, 1990; Martín and Liras, 1989). However, sequence analysis studies revealed some similarity between ACVSR and arginase, an enzyme that converts arginine to ornithine, and whose activity was shown to correlate with the biosynthesis of clavulanic acid (Penninckx *et al.*, 1974; Romero *et al.*, 1986). It was therefore postulated that (i) ACVSR plays a role in the biosynthesis of clavulanic acid and (ii) because of its physical association with ACV-synthetase, the ACVSR/ACV-synthetase complex might serve a regulatory function, that affects the biosynthesis of cephamycin at the level of ACV-synthetase.

This view was reinforced by the studies of Martín and co-workers (Romero *et al.* 1986) which showed an amino acid-dependent dissociation of these two biosynthetic pathways by *S. clavuligerus* cultures grown in a defined medium. In their studies, ornithine suppressed the biosynthesis of cephamycin but not of clavulanic acid; whereas arginine showed a concentration-dependent stimulation of clavulanic acid production and inhibition of cephamycin C biosynthesis in resting cells. It was therefore postulated that this effect by amino acid was likely mediated in some ways by the complex ACVSR/ACV-synthetase either through the modulation of enzyme activities or regulation of the expression of their corresponding genes.

Since ACVSR co-purified with ACV-synthetase, this protein would naturally co-immobilize with ACV-synthetase. The two approaches were adopted to investigate the function of ACVSR. The first involved physiological studies aimed at separating the clavulanic acid and cephamycin biosynthetic pathways according to Romero *et al.* (1986), and analysis of the activity, production and association of ACV-synthetase and ACVSR. The second aimed at separating the two proteins *in vitro* through additional chromatographic purification steps, prior to the investigation of the effect of ACVSR on ACV-synthetase. With the first approach, it was speculated that one of these two proteins would not be produced under conditions that dissociate the two pathways. These experiments were not completed as new developments on the characterization of ACVSR from electrophoretic and genetic studies suggested them to be unproductive.

However, the effect of supplemental amino acids in the two growth media, glycerol plus proline and starch plus asparagine, confirmed the suppressive effect of ornithine on cephamycin biosynthesis, as reported by Romero *et al.* (1986). According to Martín and co-workers (Martín and Liras, 1989; Romero *et al.*, 1986), the drastic inhibitory effect of ornithine on cephamycin biosynthesis is presumably mediated by its inhibition of the splitting activity that generates carbamoylphosphate and ornithine from citrulline by ornithine transcarbamylase. In this study, ornithine appears to suppress cephamycin production by repressing ACV-synthetase and IPNS biosynthesis, the two enzymes analyzed. The effect of the other amino acids on the antibiotic titre varied with the medium. In starch plus asparagine-based medium, the titres were high with arginine and glutamate, similar to those in their absence. However, in the glycerol plus proline-based medium, arginine gave the highest titre. These differences were due to the ability of asparagine to support higher antibiotic productivity than proline (Aharonowitz and Demain, 1979), overriding the effect of the individual amino acid tested in the starch plus asparagine-based medium. Aharonowitz and Demain (1979) compared several amino acids in a medium containing glycerol, as a carbon source, and found that asparagine, glutamine and arginine were better nitrogen sources for cephalosporin production than proline. This finding suggests that a careful choice of a nitrogen source to support basal growth in studies investigating the effects of amino acid is important for carrying out meaningful analyses.

4.3 Mechanistic and biochemical studies of ACVSR/ACV-synthetase system.

The studies attempting to characterize ACVSR were not conclusive. It was reasoned that any detectable novel product would either derive from the interaction of ACVSR and ACV-synthetase or from the activity of the former alone. ATP was chosen in these studies because this cofactor is not only extensively used in the reaction steps of ACV-synthetase, but also known to play an important role in the regulatory mechanism of many regulatory proteins involving phosphorylation and dephosphorylation activities (Bouret *et al.*, 1989; Ronson *et al.*, 1987; Stock *et al.*, 1989). The results as summarized indicated that (i) the formation of ^{32}P-AMP was dependent on the presence of ACV-synthetase substrate amino acids and active enzyme preparation; (ii) Ca^{+2} plus PEP increased the amount of ^{32}P-AMP formed, and the effect was due to Ca^{+2} (not shown); (iii) the formation of ^{32}P-ADP from both α-^{32}P-ATP and γ-^{32}P-ATP occurred, and was also dependent on the presence of amino acid and active enzyme preparation; (iv) the level of ^{32}P-ADP formed was highest with cysteine, slightly less with valine, and could not be detected with L-α-aminoadipate; (v) the formation of ^{32}P-ADP from γ-^{32}P-ATP in reaction mixtures supplemented with unlabelled AMP was independent of the presence of amino acids, but was stimulated in their presence and was time-dependent; and (vi) the formation of ^{32}P-ADP from γ-^{32}P-ATP in reaction mixtures supplemented with unlabelled ADP did not occur in the absence of amino acids, but in their presence a time-dependent formation of ^{32}P-ADP was observed at much higher levels than those achieved with AMP supplementation. Analysis of the reaction products of purified ACV-synthetase with ^{32}P-ATP indicated that the amino acid activation activity of ACV-synthetase does indeed proceed through an aminoacyl-adenylate intermediate with the release of PP_i, and subsequently with the formation of AMP, presumably after a thioesterification step.

Since the unusual formation of ^{32}P-ADP from ^{32}P-ATP was dependent on substrate amino acids and ACV-synthetase activity, it can be concluded that this activity was dependent on the generation of the substrates, such as AMP or PP_i. A possible explanation for these observations would be that the Mono Q purified enzyme contained an activity similar to that of an adenylate kinase (Noda, 1973), that converts AMP produced by ACV-synthetase and unused ATP into ADP as follows:

Reaction step 4.1: **γ-^{32}P-ATP + AMP <--------> ß-^{32}P-ADP + ADP**

Therefore the formation of ADP served as an indirect measure of AMP formation in the reaction mixtures. This explanation is supported by the observed differences in the amount of ADP formed in the presence of individual amino acids. This correlates with the observed differences in the rate of the ATP/PP$_i$ exchange reactions catalyzed by ACV-synthetase, which was highest with L-cysteine, followed by L-valine (Schwecke *et al.*, 1992; van Liempt *et al.*, 1989). Incubation of ACV-synthetase with L-α-aminoadipate did not result in a detection of AMP.

However, evidence against the contention that the activity responsible for ADP formation was a typical adenylate kinase was obtained from AMP and ADP supplemental studies, which indicated that the reaction step 4.1 was not freely reversible as expected for this enzyme, with an equilibrium constant of 1 (Noda, 1973). In addition, this reaction had a requirement for amino acids to form the radioactively labelled ADP from radioactively labelled ATP and unlabelled ADP.

The results obtained with the Superose 6B fraction containing ACVSR suggested the involvement of this protein in the ADP forming activity. However, on the basis of the current information about ACVSR, (Aidoo, K and S.E. Jensen, personal communication), this protein is involved in the biosynthesis of clavulanic acid and is not likely responsible for the ADP forming activity. Phosphorylation of this protein was also postulated as a possible mechanism for the formation ADP from ATP, but no evidence to support this postulate was obtained from the electrophoretic analysis of ACVSR from these reaction mixtures. Therefore, it can be speculated that the ADP forming activity was presumably due to a contaminant.

The biochemical studies of ACV-synthetase were carried out to design an efficient immobilized ACV-synthetase system. The kinetic studies indicated that L-cysteine and ATP were inhibitory at higher concentrations. The Michaelis constants determined with the purified enzyme were 6.2×10^{-4} M, 4.3×10^{-5} M, 3.8×10^{-4} M and 7.6×10^{-4} M, for L-α-aminoadipate, L-cysteine, L-valine and ATP, respectively. These differed to some extent with those reported for the crude enzyme (Jensen *et al.*, 1988). From an independent study, Zhang *et al.* (1992) reported the kinetic parameters of the purified enzyme, some of which are confirmed in this study. Consistent in these analyses, is that ACV-synthetase has the highest affinity for cysteine and the lowest for L-α-aminoadipate. The estimated Vm of ACV-synthetase of 1.7 mU/mg was lower than the values of 6.3 and 10 mU/mg reported by Zhang *et al.*, (1992) and Baldwin *et al.* (1990), respectively. The value in this study may be lower due to repeated application to the Mono Q column to ensure purity and coincident denaturation. A comparison of the stability of the enzyme stored at -75°C to that stored at 4°C showed some loss of activity at 4°C overnight. For use in kinetics studies, an ACV-synthetase stabilization cocktail which contains the components whose effect is to be studied should be avoided. Of some concern was the contamination of purified ACV-synthetase preparations with ACVSR, until this component was shown to be inactive in the system.

Other properties of ACV-synthetase studied were the effect of temperature on the catalytic rate and also the effect of pH on the stability of the enzyme. Similar studies were independently carried by Zhang *et al.* (1992) with similar findings. The effect of temperature on enzyme activity up 24°C showed a linear increase in activity with temperature. Analysis of the effect up to 40°C showed an increase in enzyme activity up to 29.5°C (Zhang *et al.*, 1992), matching the typical 1.8-fold increase in rate for every 10°C increase (Scopes, 1987). At higher temperatures, the activity fell because of increasing rate of thermal denaturation. Storage of the enzyme at various pH values, ranging from 5.0 to 9.0, demonstrated that ACV-synthetase was most stable at pH 7.5 and 8.0. Drastic loss in activity occurred at pH 5.0.

4.4 Immobilized enzyme processes for the production of ACV.

The use of immobilized enzymes in single step conversions of ß-lactam biosynthetic intermediates is not only an attractive alternative to laborious chemical processes, but also a promising approach for the synthesis of novel ß-lactams using substrate analogs. The development of an immobilized ACV-synthetase reactor for the production of gram quantities of ACV *in vitro*, was a follow-up to a previous report on the immobilization of the partially purified enzyme from *S. clavuligerus* by Jensen *et al.* (1989). In those studies, ACV-synthetase immobilized on the anion-exchange resin DEAE-Trisacryl was shown to carry out repeatedly the synthesis of ACV, but after six uses the enzyme lost 70% of the initial activity. In addition the amount of product formed was quite low, in the range of 25 μM, representing only 2.5% conversion of the limiting substrate cysteine after 4 h of operation.

Initial attempts were made to covalently immobilize ACV-synthetase onto porous glass beads according to the "glutaraldehyde linker" method (Weetall 1976) and the "glutaraldehyde crosslinker" method (Kadima and Pickard 1990), methods that were successfully used to immobilize the fungal enzyme chloroperoxidase. The binding of ACV-synthetase to the carrier was observed with the second method, but the resulting immobilized enzyme material was not active. Yet the enzyme remaining in solution was quite stable during the procedures.

The ionic binding of ACV-synthetase to DEAE-Trisacryl was achieved with purified enzyme. Jensen *et al.* (1989) estimated the loading capacity of DEAE-Trisacryl to be about 81 to 94 mg protein/g of damp resin and this was not exceeded in this study, as indicated by the complete binding of the protein exposed to the resin. But the immobilized preparation had only 4.4% of the specific activity of an equivalent amount of soluble enzyme. Analysis of the catalytic activity of the enzyme immobilized on DEAE-Trisacryl by Jensen *et al.* (1989) suggested also that the specific activity of the bound enzyme decreased from 94 x 10^{-3} to 4.6 x 10^{-3} mU/mg of protein, corresponding to a 95% reduction. This decrease in the activity of the immobilized enzyme was partly due to mass transfer limitation. However, some improvement in the catalytic rate was observed with increasing agitation. It is unlikely that increased aeration was responsible for the observed increase in activity, for the enzyme in the reactor that was not shaken retained a larger fraction of its activity during the same incubation period.

The low specific activity of the ionically bound and lack of activity of the covalently bound enzymes were likely due to conformational and steric effects (Kennedy and Cabral, 1983). Interference of the binding to the support with the proper functioning of the enzyme of the size of ACV-synthetase, either from occlusion of the active site or restriction of enzyme flexibility, can negatively influence the activity the immobilized enzyme product.

In order to reach the initial objective of producing large quantities of ACV, the development of an alternative and efficient immobilized enzyme process was necessary. A third procedure was considered which was based on physical restraint of the enzyme in a selectively permeable reactor. This procedure presented a number of advantages over the other methods investigated. First, the large size of ACV-synthetase permitted the repeated use of the enzyme within the reactor without any prior chemical coupling to a matrix, and the removal by filtration of the low molecular weight products and unused substrates in a semi-continuous fashion. Second, when using the partially purified enzyme, this system enabled the exploitation of the stimulating ability of the endogenous cellular ATP regenerating components and also minimized loss and handling of the enzyme associated with extensive purification procedures. Third, enzymic activity was not affected by mass transfer limitations and loss of activity due to improper coupling to the support. And fourth, the ambient atmosphere could be controlled to minimize oxidation by air. An additional advantage of the use of a modified Amicon cell as a reactor for immobilized enzymes is that both entrapped-soluble and support-bound enzymes can be studied under similar conditions and their properties compared.

ACV-synthetase is believed to have sulfhydryl groups essential for its activity, which are subject to oxidation by oxygen. To minimize the loss of enzymic activity by way of this oxidation, reducing agents such as dithiothreitol, dithioerythritol, or ß-mercaptoethanol , were usually added to the enzyme preparation. However, since the susceptibility to damage increases with temperature and during prolonged incubation at ambient temperatures, an alternative mechanism to the use of reducing agents was to remove oxygen from the reactor to increase the lifetime of the enzyme. This was achieved by replacing air with a nitrogen atmosphere in the enzyme reactor at room temperature. After several repeated uses, the enzyme retained 35 to 40% of its initial operational activity after 24 h. This system stabilized both the enzyme and the product, ACV. The disappearance of ACV from the reaction mixtures incubated in air was found to be due to the oxidation of ACV, resulting in the formation of a dimer. This structure could however, be reduced with DTT. Part of the enzyme stability during operation at room temperature was probably due to incubating in the presence of substrates than in their absence. A similar improvement of gramicidin S synthetases stability was observed in the presence of substrates, resulting in the persistence of gramicidin S production for several hours at 37°C (Demain and Wang, 1976; Hamilton *et al.*, 1973).

The physically-confined ACV-synthetase was used repeatedly to produce ACV, and it converted up to 25-30% of the amount of cysteine added to the reactor, with all other components being in excess. Immobilization of a cephalosporin acetylesterase from *Bacillus subtilis* by containment within an ultrafiltration device has been reported (Abbott *et al.*, 1976). This enzyme which deacylates 7-aminocephalosporanic acid solutions containing 4 to 24 mg/mL of this compound was reused 20 times over an 11 day span. In this study, the immobilized ACV-synthetase was reused 5 times, then the specific productivity began to decrease. The loss of activity is likely due to inactivation or adsorption of the enzyme to the membrane and walls of reactor vessel. No estimation of the protein recovery at the end of the operation was carried out to analyze the reason for the decreased productivity. During the purification of ACV-synthetase, Schwecke *et al.* (1992) observed loss of activity due to binding of enzyme to ultrafiltration membranes.

Production of ACV at levels greater than 0.25-0.30 mM could not be achieved. Many experiments were carried out trying to determine the factors, different from enzyme stability, that affected ACV production levels. These included: enzyme amounts added to the reactor and the buffer system components, product inhibition and exogenous enzymes, and incremental supplementation of substrates, particularly the limiting cysteine. It was found that the amount of enzyme added to the reaction mixture was important, and best kept low for achieving the highest specific productivity. Since the enzyme was prepared in buffer system containing glycerol, the effect of glycerol on the catalytic rate of the enzyme was also analyzed, and found to have a negative effect. ACV itself at concentrations of up to 137 μM did not have any effect on enzyme activity. But higher concentrations were not tested due to technical difficulties with the assay system, since the increase in background levels of ACV did not permit an accurate determination of the amount of product formed from catalytic activity. It is however unlikely that ACV is exerting product inhibition on the enzyme, since some other enzyme preparations were found to be less efficient in producing ACV.

A number of reaction products from ATP utilization were found to inhibit the enzyme to varying degrees. Of these, the enzyme was most affected by AMP, but only at non-physiological concentrations. The addition of adenylate kinase, adenylic acid deaminase, or inorganic pyrophosphatase to the reaction mixture did not improve the conversion efficiency, suggesting that AMP and PP_i were not likely the primary cause for the observed limitation to substrate conversion at 25-30%. Because of the inability to determine the factors that limited conversions no strategy was developed to overcome this phenomenon.

The economical feasibility of this process for the production of ACV depends on the improvement of enzyme stability and substrate conversion efficiency, the reduction of costs associated with enzyme production, purification and immobilization. The immobilization procedure of physical confinement of the enzyme in a selectively permeable reactor was a simple procedure. Based on 25% conversion in 3 h and stability of the enzyme as shown in Table 3.6, a 1-L reactor would produce 91 mg ACV, and in 24 h about 411 mg ACV could be produced by the same batch of enzyme. A preliminary reagent cost analysis for a 1-L reaction mixture is summarized in Table 4.1, based on the 1992 pricing from Aldrich catalogue.

Bacterial contamination is a potential problem for long-running room temperature experiments and maintenance of sterile conditions may be required for preparative purposes. Sodium azide has been found to be a useful antibacterial agent in an ACV-synthetase assay. Preincubation of the enzyme with up to 0.1% for 20 min had little or no effect on enzymic activity. Physical loss of the reaction volume was also observed as a result of the geometry and can be minimized by using a smaller size (10-mL) reactor. In these studies, a 5 to 6 mL-reaction mixture was used in a 50-mL-Amicon. The reaction mixture volume was kept small to minimize the cost of the reagents. Therefore the use of smaller filtration units would minimize sample volume variations and improve reproducibility.

4.5 *In vitro* reconstitution of α-aminoadipate binding domain activity of ACV-synthetase.

This research project involved the production and characterization of the recombinant putative domain-1 polypeptide of ACV-synthetase. The main objective was to confirm the biological function of ACV-synthetase domain-1. Since the recombinant polypeptide was produced in an inactive aggregated form, efficient recovery, solubilization and renaturation of this polypeptide from inclusion bodies became also important aspects of the project.

The heat-inducible expression system of *E. coli* pOW409/JM109 was engineered for the expression of ACV-synthetase domain-1 polypeptide, but the protein was produced as inactive inclusion bodies. The renaturation procedure used in previous studies was based

Table 4.1 Reagent cost-analysis of ACV production by immobilized ACV-synthetase reactor.

Reagent [(1)]	Amount used in 1-L reaction mixture (g)	Cost per 1-L reaction mixture (US $)
Na_2ATP (2 mM)	1.10	5.72*
$MgCl_2$ (6 mM)	1.22	11.28
DTT (2 mM)	0.03	0.31*
Tris (100 mM)	3.87	0.18*
PEP (5 mM)	1.03	95.38
L-AAA (5 mM)	0.81	45.76
L-cysteine (1 mM)	0.18	0.19*
L-valine (5 mM)	0.59	0.54*
Total		159.31

(1) The reagents used in the ACV-synthetase assay mixture at the final concentration shown in parentheses.

* The price has been estimated from the bulk price, which is less than the listed price per gram of reagent.

on a rapid removal of urea from a concentrated solution of denatured protein, using a desalting column. Those studies indicated that the renatured domain-1 polypeptide preparation quickly lost its ability to bind ^{14}C-amino acids and that this binding activity was not specific. Because these results were not consistent, convincing evidence was still needed to demonstrate the specificity of the interaction between ACV-synthetase domain-1 with only one of the three substrate amino acids of ACV-synthetase.

Previous studies revealed a problem with the renaturation step. Following the removal of urea by gel filtration, the renatured domain-1 polypeptide solution turned turbid, suggesting that the solubilization of the aggregates under the conditions used was incomplete, or reaggregation of the unfolded protein was taking place quite rapidly, or both. The clarification procedure used to remove insoluble protein, involving a 5- to 10-min centrifugation step at low speed, was only effective for large precipitable aggregates. Therefore, if the solution of the presumed renatured protein contained soluble aggregates, which were still associating and eventually forming precipitable material with time, this would explain the rapid loss of binding activity that was observed during short storage. Alternatively it may be a problem of protein stability. Stability of the refolded polypeptide could not be analyzed in these preliminary studies, as the system was not well understood. However, the lack of specific binding activity could result from entrapment of the labelled amino acid in the aggregating protein.

Two aspects of the characterization of ACV-synthetase domain-1 polypeptide, were first investigated: (i) increasing the yield of the domain-1 polypeptide through increase of biomass of the induced culture, and (ii) evaluation of the methods available to optimize the solubilization and refolding of domain-1 polypeptide.

A number of media were investigated for growth, and the Tabor medium was found to support high biomass production. The induced cells produced the domain-1 polypeptide as inclusion bodies, which represented 21% of the total cellular protein, though the estimation of protein from inclusion bodies varied with the preparation of the aggregates. Prior solubilization of inclusion bodies in urea gave estimates that were at least two-fold higher than when this step was omitted, suggesting that the aggregated protein was not completely accessible to the protein assay reagent, and harsh denaturing conditions were required to disrupt the strong noncovalent forces holding the polypeptide structures together (Mitraki and King, 1989). Most of the contaminating proteins in the inclusion bodies were likely cell envelope associated proteins (Bowden *et al.*, 1991; Hartley and Kane, 1988; Marston, 1986). However, the same protein banding profile was observed even when the cell-free extract was prepared from sphaeroplasts and the inclusion bodies were treated with the detergent Triton X-100 (Lin and Chen, 1991).

Successful renaturation of a protein that has accumulated into inclusion bodies requires the complete unfolding of the polypeptide (Marston, 1986; Mitraki and King, 1989). This can be done in a number of ways using physical or chemical reactions (Tanford, 1968). The protocol used in our laboratory to solubilize the inclusion bodies involved chemical denaturation with urea. Systematic studies of the parameters that influenced functional renaturation of domain-1 polypeptide were carried out using two methods. The first approach involved the estimation of the amounts of protein in the soluble fraction of the denaturant-treated inclusion bodies by protein assay, SDS-PAGE analysis, or both (Bowden *et al.*, 1991; Brown *et al.*, 1992). The drawback of this approach was the inability to distinguish fully unfolded polypeptides from incompletely unfolded intermediate-size aggregated polypeptides, which could not be separated by low speed centrifugation. In addition, increasing the concentration of the denaturant resulted in a viscosity increase (Tanford, 1968), requiring increased centrifugation to sediment the aggregates (Scopes, 1987).

The second approach was based on spectral analysis of the unfolded polypeptide in the UV/visible wavelength region, as described earlier (section 3.4.2). Spectroscopic methods such as circular dichroism spectroscopy, fluorescence spectroscopy, and quasy-elastic light scattering spectroscopy are better tools for the analysis of conformations and association forms of polypeptide molecules (Cleland and Wang, 1990b; Elove *et al.*, 1992; Javor *et al.*, 1991), but these were not routinely available. Alternatively, a single absorption reading at 320-340 nm, which gave the largest difference in readings between the aggregated and denatured polypeptide samples, was used in the analysis of the two states (Jaenicke and Rudolph, 1989). Spectral analysis was the preferred method, since it was able to detect changes in the baseline arising from variations in the sample. Spectral analysis had the added advantage of being rapid, convenient and useful for the analysis of the state of renaturation.

Scanning the absorption spectrum of the aggregated polypeptide in the absence and presence of denaturant revealed profile differences, characteristic of the aggregated and solubilized forms of the polypeptides. The soluble denatured polypeptide absorbed strongly at 280 nm, due to aromatic ring absorption. The aggregated polypeptide suspension absorbed in both the UV and visible regions, due to light scattering.

The effect of the denaturant concentration on the solubilization of domain-1 aggregates was analyzed with urea and the effective concentration for the solubilization of the aggregates was estimated to be at least 8.0 M, typical concentrations used for the solubilization of inclusion bodies and unfolding of proteins (Mitraki and King, 1989; Tanford, 1968). To regain biological activity, the unfolded polypeptide was then renatured.

The proper refolding of unfolded polypeptide *in vitro* is a much more complex process than the solubilization of aggregated polypeptides, a reflection of the conditions and the polypeptide folding aids which are required for the success of this process *in vivo* (Ellis and van der Vies, 1991; Fischer and Schmid, 1990; Gething and Sambrook, 1992; Langer *et al.*, 1992; Nilsson and Anderson, 1991). Numerous approaches have been used to maximize the recovery of properly folded polypeptide from inclusion bodies. The majority of methods are designed without the aid of exogenous proteins, such as the molecular chaperones (Creighton, 1991; Brown *et al.*, 1992; Wiech *et al.*, 1992; Zeilstra-Ryalls *et al.*, 1991), and have been mainly concerned with the manner in which the denaturant is removed from the polypeptide sample in the presence of a stabilizing buffer (Cleland *et al.*, 1992; Creighton, 1986; Mitraki and King, 1989).

The gel filtration method was used previously, but with limited success. One alternative method involved the removal of urea by dialysis or quick dilution in the presence or absence of a co-solvent. The rationale behind this method is that as urea is removed, the polypeptide slowly adopts its native conformation, presumably via "molten globule"-like folding intermediates. For some proteins, such as *E. coli* tryptophanase and horse muscle phosphoglycerate kinase (London *et al.*, 1974; Mitraki *et al.*, 1987), incubation of denatured polypeptides at intermediate denaturant concentrations stabilized such folding intermediate species, leading to reaggregation and poor recovery of active enzymes. Therefore it is necessary to quickly reduce the concentration of urea, a procedure that also reduces the concentration of the polypeptide chains in the folding reaction (Mitraki and King, 1989; Zettlmeissl *et al.*, 1979). However for other proteins, such as the monomeric alpha-helical bovine growth hormone and bovine carbonic anhydrase B, incubation at certain intermediate denaturant concentrations, appears to stabilize an intermediate folding species that would ultimately fold properly during the final stage of renaturation under denaturant-free conditions (Cleland and Wang, 1990b; Doligh *et al.*, 1984; London *et al.*, 1974; Mitraki *et al.*, 1987; Semisotnov *et al.*, 1987). Alternatively, a co-solvent such as polyethylene glycol, that interacts with exposed hydrophobic surfaces of folding intermediate species, has been shown to prevent reaggregation (Cleland and Wang, 1990a). The renaturation of ACV-synthetase domain-1 polypeptide was attempted under a variety of dialysis, dilution and PEG-co-solvent conditions. In all cases, the renatured polypeptide preparation showed evidence of reaggregation. This reaggregation could not be reduced by lowering protein concentration of the reaction. It was concluded that these approaches were not suitable for the renaturation of ACV-synthetase domain-1 polypeptide.

The renaturation of proteins adsorbed reversibly to ion-exchange resins has been demonstrated (Creighton, 1986). The concept for the renaturation by ion-exchange chromatography is attractive for a number of reasons: (i) the protein loaded on the column need not to be concentrated in a small volume, as with gel filtration, (ii) the refolding conditions of the protein bound onto the column could be controlled by gradual changes of the solvent systems, using a series of gradients from denaturing to refolding conditions, and (iii) the refolding process was likely to occur much more slowly as the polypeptide desorbs from and readsorbs to the column during elution with high salts. However, attempts to refold domain-1 aggregates bound on DEAE-Trisacryl or Mono Q columns also failed. Only small amounts of protein could be eluted from the column, even with a high concentration of salts. Investigation of alternative ion-exchange resins indicated that the solubilized domain-1 aggregates bound exclusively to anion-exchange resins, but still the protein could not be recovered. Non-denatured inclusion bodies could be directly exposed to the ionic support, and desorbed from the support with high salt, but the desorbed protein was still in its aggregated form. This method has been reported to result in the refolding of some proteins (Hoess *et al.*, 1988), but was not successful here. This method is rather difficult to conceive: it is believed that the electrostatic forces of ionic groups on the resin are strong enough to disrupt certain aggregated polypeptides and bind the separated polypeptide units. The desorption by high salt of the resin-bound polypeptide would then yield properly folded protein.

Analysis of the solubilized inclusion bodies by SDS-PAGE, following an attempt to purify the domain-1 polypeptide by gel filtration under denaturing conditions in 7.2 M urea, indicated that the denaturation conditions used to this point did not result in a complete dissociation and unfolding of the aggregated polypeptides. This finding was unexpected, for solubilization of aggregates was evident from spectral analysis and visually, as the solution turned from milky white to clear upon addition of urea. Achieving the proper refolding of ACV-synthetase domain-1 polypeptide *in vitro* evidently was not a straight forward process. There were many possible explanations, and three of these are outlined here. First, the refolding of ACV-synthetase domain-1 *in vitro* was not possible because the incomplete polypeptide sequence could not form a stable tertiary structure. Yet the ornithine activating fragment of gramicidin S synthetase 2 from *B. brevis* has been cloned and expressed in *E. coli*, yielding a product with biological activity (Krause *et al.*, 1985). Second, ACV-synthetase domain-1 could refold as a discrete native domain, but the absence of the other two domains leads to the formation of a tertiary structure with exposed hydrophobic surfaces, that are normally involved in the attachment to other domains in the native enzyme. Where the aggregation reaction dominates the *in vitro* refolding process, its inhibition becomes crucial for regaining activity (Mitraki and King, 1989). Such a case is the refolding of the monomeric, but two domain enzyme, rhodanese. The refolding reaction appears to generate an intermediate with quasi-structured domains, carrying exposed hydrophobic surfaces. Horowitz and co-workers used detergents to inhibit the aggregation reactions and successfully renatured it in the presence of lauryl maltoside (Horowitz and Criscimagna, 1986; Tandon and Horowitz, 1986). Preventing reaggregation of the exposed hydrophobic surfaces with solvent system that minimizes hydrophobic interactions, such as the water miscible solvents: dimethylsulfoxide and N,N-dimethylformamide to keep the domain-1 polypeptide units separate was a possibility. Alternatively, these surfaces could be masked with a reagent that interacts with hydrophobic surfaces.

The third explanation was simply that, the solubilization and renaturation conditions were not optimized. The inability to purify the unfolded domain-1 polypeptide by gel filtration provided some support for this explanation, particularly with respect to the solubilization step. The latter two possibilities were investigated first.

A number of changes to the reaction conditions were necessary for the successful refolding of the domain-1 polypeptide to occur. These included an increase in the concentration of the urea to 9.0 M, a decrease in the glycerol concentration from 20% to 5% (v/v) during the denaturation step; the replacement of MOPS buffer, pH 7.5 with Tris buffer, pH 8.5 during the refolding, and incubations carried out overnight at 10°C, with protein concentrations of less than 0.1 mg/mL. The refolded polypeptide was further treated with 0.05% SDS before concentration. The most important changes were the increase in urea concentration and the reduction in glycerol concentration. Gekko and Ito (1990) had demonstrated competing solvent effects of polyols and guanidine hydrochloride on protein stability. It was probable that in the presence of glycerol (20%), the effective concentration of the denaturant necessary to completely solubilize the aggregates was higher than in the absence of glycerol. SDS is an amphipathic detergent which at low concentration can react with hydrophobic surfaces on certain native proteins without denaturing the protein, and some proteins remain unaffected with the preparation of cell-free extract in the presence of low amounts of SDS (Cooper, 1976). An interesting observation was that the ACV-synthetase inclusion bodies could also be solubilized with a 0.05% SDS solution to a form able to enter polyacrylamide gels. These observations suggest that SDS has likely masked the hydrophobic reactive surfaces on the refolded domain-1 polypeptide.

Evidence for the proper folding of ACV-synthetase domain-1 was demonstrated with the spectrum of the renatured domain-1 polypeptide which showed absorption at 280 nm, which is typical of many other proteins, including bovine gamma globulin and chloroperoxidase. In addition the renatured polypeptide could be observed by gel electrophoresis under both denaturing and non denaturing conditions, a situation not previously realized. Electrophoresis of the renatured domain-1 polypeptide sample under "pseudo non-denaturing" conditions, in the presence of 0.05% SDS, but without ß-mercaptoethanol and heating, showed that the domain-1 polypeptide migrated slightly slower than under denaturing conditions. This was likely due to retention of its tertiary structure and slight increase in the surface negative charge under these conditions.

The biological activity of the refolded domain-1 polypeptide was investigated with ^{14}C-labelled amino acids. Reports on the characterization of ACV-synthetase from *A. nidulans* (MacCabe *et al.*, 1991), *C. acremonium* (Hoskins *et al.*, 1990; Gutiérrez *et al.*, 1991), *N. lactamdurans* (Coque *et al.*, 1991) and *P. chrysogenum* (Díez *et al.*, 1990; Smith *et al.*, 1990) indicated that this enzyme consisted of three functional domains. The hypothesis proposed for the function of the putative ACV-synthetase domain-1 was that this polypeptide is one of the three domains of ACV-synthetase and may contain the binding site for one of the three substrate amino acids. On the basis of the upstream location of the DNA fragment encoding this polypeptide in the sequence of ACV-synthetase gene, it was therefore presumed to be the putative domain for L-α-aminoadipate, the amino acid occupying the first position in the synthesis of the tripeptide ACV. Specific binding with only L-α-aminoadipate was expected and this was observed in this study with the polypeptide renatured under a variety of conditions, except in the presence of Triton X-100. This lack of binding activity with Triton X-100 renaturation, was believed to be due to the denaturation of the refolded protein within the detergent which appeared to form a separate phase during the washing of the sample with TCA.

These results indicated that despite the apparent association of the refolded domain-1 monomer, the resulting protein complex still retained specific binding activity, since the presence or absence of co-solvent, SDS or NaCl did not result in significant differences in the level of binding of the amino acid detected. The scheme outlined in Figure 4.1 shows an hypothetical model of the folding pathway of domain-1 polypeptide into a native molecule capable of binding L-AAA. However, due to the unavailability of the other two domains and inter-domain association via hydrophobic surfaces, as may be the case in the native ACV-synthetase, the domain-1 molecules can associate via these exposed surfaces with each other to form high-molecular weight protein material that cannot be resolved in a non-denaturing polyacrylamide gel. Treatment of these native domain-1 polypeptides with low-concentration SDS, would mask such exposed hydrophobic surfaces and prevent their reassociation.

The identification of the functional L-α-aminoadipate binding domain of ACV-synthetase achieved in these studies, constitutes the first evidence at the protein level in support of a reaction mechanism involving separate domains for the activation of individual substrate amino acids by this enzyme. ATP-pyrophosphate exchange activity, as well as

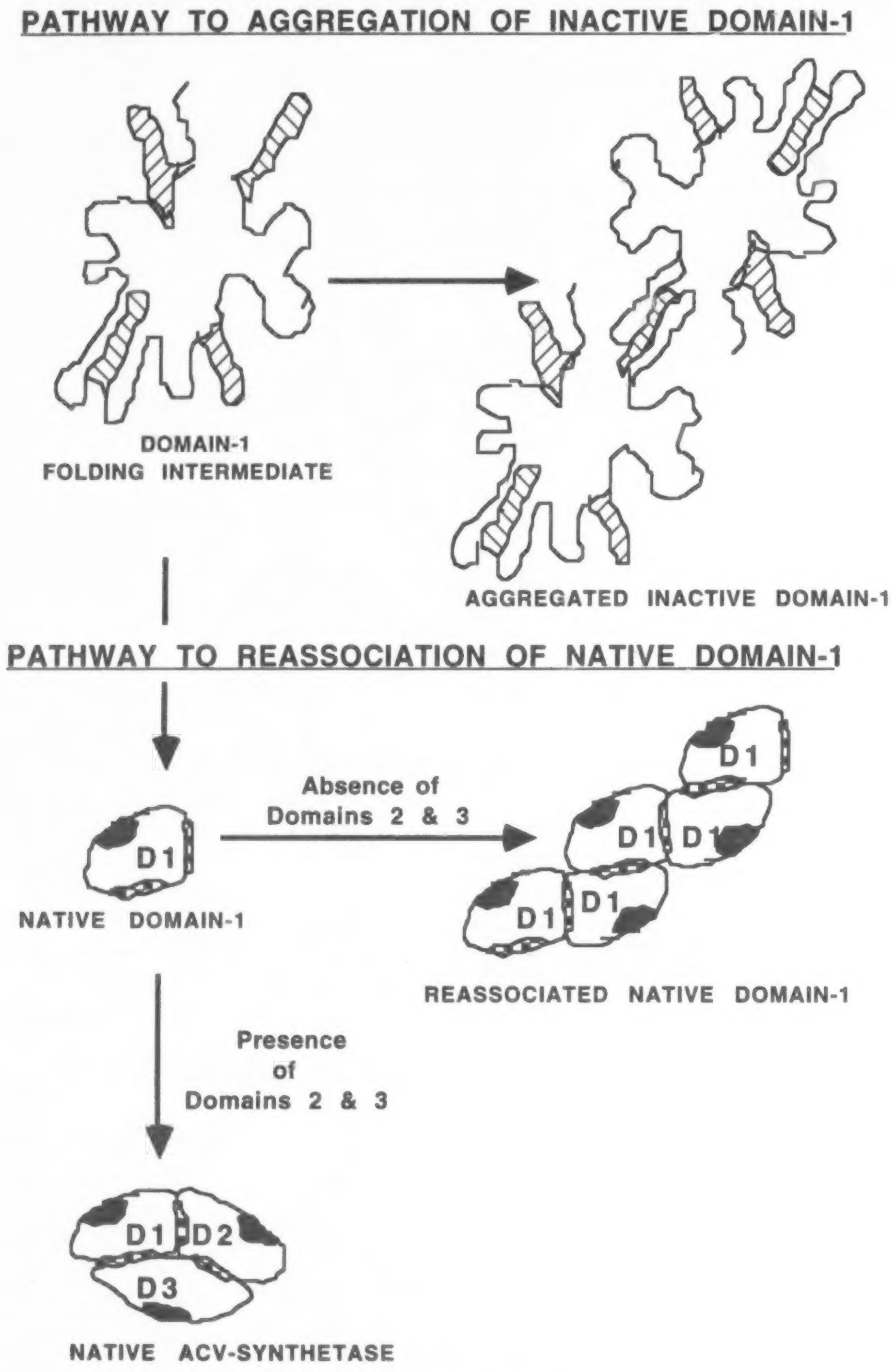
Figure 4.1 Scheme for the refolding of the domain-1 polypeptide of ACV-synthetase. Two pathways are shown.

Top: When the conditions in the refolding reaction mixture are not suitable for the functional renaturation of the unfolded polypeptides, improper intra- and intermolecular interactions of the domain-1 folding intermediates can lead to the formation of inactive aggregates. Shown also are the hypothetical secondary structures already formed in the folding intermediate species, but no final tertiary structure has formed yet.

Middle: Under appropriate conditions, functional renaturation would occur, leading to the formation of native domain-1.

Bottom: In the absence of domains 2 and 3, however, self-association of properly folded domain-1 molecules can occur via exposed hydrophobic surfaces, that are covered through the attachment of the other two domains in the native ACV-synthetase.

Shaded areas represent the native amino acid binding site of each native domain and the hydrophobic surfaces for inter-domain attachment.


PATHWAY TO AGGREGATION OF INACTIVE DOMAIN-1
DOMAIN-1
FOLDING INTERMEDIATE
AGGREGATED INACTIVE DOMAIN-1
PATHWAY TO REASSOCIATION OF NATIVE DOMAIN-1
D1
NATIVE DOMAIN-1
Absence of
Domains 2 & 3
D1
D1
D1
D1
D1
REASSOCIATED NATIVE DOMAIN-1
Presence
of
Domains 2 & 3
D1
D2
D3
NATIVE ACV-SYNTHETASE

^{14}C-amino acid binding activities, have been reported for the native enzyme (Schwecke *et al.*, 1992; van Liempt *et al.*, 1989). Such studies however, give no information on the structural organization of ACV-synthetase for the catalysis of the amino acid activation reactions. In the gramicidin S synthetase system from *B. brevis*, the domain responsible for the activation of ornithine had been isolated and characterized (Krause *et al.*, 1985). The data presented here suggest a similarity between these two enzymes in their mechanism of action, the multienzyme thiotemplate mechanism (Aharonowitz *et al.*, 1993b), which may extend to their structural organization.

4.5 Future studies

The immobilization of ACV-synthetase by physical confinement was a suitable process for the design of a reusable ACV-synthetase reactor for ACV production. A number of additional studies may be carried out to achieve the total synthesis of novel ß-lactams using a series of immobilized enzyme reactors of all the biosynthetic enzymes. This arrangement is necessary for improving individual steps and minimizing the inhibitory effects of the reagents from one reaction to another, as in the case of L-cysteine and L-α-aminoadipate on DAOC synthase (Castro *et al.*, 1985; Jensen *et al.*, 1989), or oxygen on ACV-synthetase. Favoring the oxidation reaction of the product ACV may be used to purify ACV from other low molecular-weight reaction mixture components by a number of ways. One such process includes the use of an activated thiol-solid phase support, such as agarose glutathione-2-pyridyl disulphide (Smith and Hider, 1988). Such a resin containing available sulfhydryl groups might be used to bind ACV from the reaction mixture under oxidizing conditions: prolonged incubation to form a disulfide derivative under forced air, followed by elution with buffer containing DTT, resulting in the purification of ACV. The problem with this approach would be defining the conditions that would only promote disulfide bridge formation between free ACV and bound thiol groups and not between ACV molecules to form bis-ACV. Alternatively high performance size exclusion chromatography can be used to purify the product.

These studies have achieved functional renaturation of the domain-1 polypeptide. Further studies can attempt its purification under denaturing conditions, possibly at room temperature and in the presence of low concentration of SDS. With purified polypeptide, X-ray crystallography analysis of ACV-synthetase domain-1 may be the next step to determine its structure. Such analysis would provide useful information on the organization the peptide synthetase reaction center, and how domains are assembled in the native enzyme.

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